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Identification of T-Cell Epitopes in Nonstructural Proteins of Foot-and-Mouth Disease Virus

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Porcine T-cell recognition of foot-and-mouth disease virus (FMDV) nonstructural proteins (NSP) was tested using in vitro lymphoproliferative responses. Lymphocytes were obtained from outbred pigs experimentally infected with FMDV. Of the different NSP, polypeptides 3A, 3B, and 3C gave the highest stimulations in the in vitro assays. The use of overlapping synthetic peptides allowed the identification of amino acid regions within these proteins that were efficiently recognized by the lymphocytes. The sequences of some of these antigenic peptides were highly conserved among different FMDV serotypes. They elicited major histocompatibility complex-restricted responses with lymphocytes from pigs infected with either a type C virus or reinfected with a heterologous FMDV. A tandem peptide containing the T-cell peptide 3A[21–35] and the B-cell antigenic site VP1 [137–156] also efficiently stimulated lymphocytes from infected animals in vitro. Furthermore, this tandem peptide elicited significant levels of serotype-specific antiviral activity, a result consistent with the induction of anti-FMDV antibodies. Thus, inclusion in the peptide formulation of a T-cell epitope derived from the NSP 3A possessing the capacity to induce T helper activity can allow cooperative induction of anti-FMDV antibodies by B cells.

The identification and characterization of T-cell epitopes is important for understanding protective immunity against pathogens mediated by $CD8^+$ lymphocytes as well as $CD4^+$ lymphocyte activities (3). Recognition of T-cell epitopes by lymphocytes from different species and individuals is restricted by the polymorphism of the major histocompatibility complex (MHC) molecules, which are responsible for the presentation of foreign antigens by antigen-presenting cells (25). Therefore, the identification of T-cell epitopes capable of inducing an effective response, while being widely recognized by MHC alleles frequent in natural populations of host species, is a problem for the development of new vaccines, particularly those based on synthetic peptides (41).

Foot-and-mouth disease virus (FMDV) is a picornavirus that produces a highly contagious disease of cloven-hoofed farm animals (36). The FMDV particle contains a positive-strand RNA molecule of about 8,500 nucleotides, enclosed within an icosahedral capsid comprising 60 copies each of four virus proteins VP1 to VP4 (reviewed in reference 4). The genome encodes a unique polyprotein from which the different viral polypeptides are cleaved by viral proteases (46), including nine different mature nonstructural proteins (NSP). Each of these NSP, as well as some of the precursor polypeptides, are involved in functions that are relevant to the virus life cycle in infected cells (reviewed in reference 37). FMDV shows a high genetic and antigenic variability, which is reflected in the seven serotypes and the numerous variants described to date (reviewed in reference 22). FMD control is mainly implemented by using chemically inactivated whole virus vaccines (reviewed in reference 5). Viral infection and immunization with conventional vaccines usually elicit high levels of circulating neutralizing antibodies, which correlate with protection against the homologous and antigenically related viruses (54). However, chemically inactivated vaccines have a number of disadvantages. Among these are the requirement for a cold chain to preserve capsid stability, the need for periodic re vaccination with virus strains antigenically similar to the circulating viruses, and the risk of virus release during vaccine production (5). These limitations have led to the search of alternative, safe immunogens.

The antigenic structure of the virus recognized by B lymphocytes has been characterized in detail (reviewed in references 10 and 30), from which the main B-cell epitopes are seen to be located in defined structural motives exposed on the surface of the capsid (2). A region located in the G-H loop, at positions 140 to 160 of capsid protein VP1, has been identified as the main continuous viral epitope recognized by host B lymphocytes to produce neutralizing antibodies (6, 9). Peptides spanning VP1 residues 140 to 160 retain reactivity with neutralizing monoclonal antibodies (MAbs) and induce neutralizing antibodies when used as immunogens (6, 9, 10, 30). However, VP1, either purified from virions or expressed in different systems, has been shown to be a poor immunogen in terms of production of neutralizing antibodies and protection, probably due to an unproper exposure of site A (9, 22). The B-cell site A has been widely used as an immunogenic peptide (reviewed in reference 9). DiMarchi et al. (21) reported protection against virus challenge infection in cattle immunized with a peptide in which the VP1 residues 140 to 160 were colinearly synthesized with those corresponding to VP1 residues 200 to 213. However, further results involving larger number of animals have shown that these peptides afford limited protection

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in natural hosts (51). One of the limiting factors of peptide vaccines may be the absence of T-cell epitopes capable of inducing the T-cell help required in cooperation with immune B lymphocytes for the production of specific antibody (15, 41, 48). The induction of anti-FMDV antibodies is T cell dependent (18). In recent years, several T-cell epitopes recognized by cattle and swine lymphocytes have been identified in the FMDV capsid proteins (7, 17, 39, 55). Inclusion of one of these T-cell epitopes identified in VP1 residues 21 to 40 in a tandem peptide with the B-cell site A has been shown to overcome individual nonresponsiveness of cattle to peptide A (17). However, the recognition of this and other T-cell epitopes identified in FMDV capsid proteins is significantly restricted by the MHC polymorphism of the host species (24, 26, 39), and their potential to improve synthetic vaccines is, therefore, limited. Yet T-cell epitopes relevant to the induction of a protective response have also been described in the NSP of several viruses (13, 27). Consequently, an extension of analysis of T-cell epitopes to FMDV NSP offers the possibility to broaden the repertoire of viral epitopes recognized by host immune defenses. In addition, the low degree of amino acid variation in NSP among different FMDV serotypes should enable the identification of T-cell epitopes recognizable in a heterotypic manner, an important requirement for inclusion in a synthetic vaccine against this virus.

We therefore analyzed the heterotypic lymphoproliferative responses against different NSP, using lymphocytes from FMDV-infected pigs. For the NSP 3A, 3B, and 3C, which consistently induced higher responses, overlapping synthetic peptides were employed to identify MHC class II-restricted T-cell sites. A tandem peptide including the B-cell antigenic site VP1[137–156] colinearly synthesized with one of this T-cell peptides, corresponding to 3A residues 21 to 35, was capable of eliciting significant levels of serotype-specific antiviral activity, a finding consistent with the induction of anti-FMDV antibodies.

MATERIALS AND METHODS

Experimental infections. Ten Landrance × Large White pigs, 3 to 4 months old (obtained from different litters), were inoculated by intradermal injection of 10^5 PFU of a type C FMDV isolate (C-S8) into the coronary matrix of the foot. All of the animals were free of previous FMD contact, as confirmed by the absence of detectable anti-FMDV antibodies in the serum. At 120 days postinfection (p.i.), all pigs were reinfected with 10^5 PFU of a type O FMDV isolate (O-BFS). In all cases, infections and reinfections resulted in fever and lesion (vesicle) development from day 2 p.i. One animal inoculated with phosphate-buffered saline (PBS) was used as a negative control.

Fusion proteins. Different NSP, as well as the VP1 capsid protein from a type O FMDV isolate (O1Kb), were expressed in *Escherichia coli*, as fusion proteins with the N-terminal part of MS2 polymerase (49). Figure 1A shows the viral polypeptides used in this study. Proteins were obtained from heat-induced bacterial cultures by sonication and purification using 7 M urea. Viral polypeptides were tested in lymphoproliferative assays as described elsewhere (39). Briefly, 20 μg of each polypeptide was separated using discontinuous 12% polyacrylamide gel electrophoresis (wt/vol) gels and transferred onto nitrocellulose sheets. The different viral polypeptides were identified by Western blotting using protein-specific rabbit antisera (38). Nitrocellulose fragments containing individual viral polypeptides, as well as control fragments containing nonviral proteins, were solubilized in dimethyl sulfoxide as described earlier (1) and used in the proliferation assays.

Peptide synthesis. A total of 83 pentadecapeptides covering the entire 3ABC precursor sequence of FMDV isolate C-S8 (29, 53) and overlapping each other by 10 residues were prepared. Their sequences and locations on the corresponding NSP are shown in Table 1. The peptides were synthesized in N-terminal free, C-terminal carboxamide form by 9-fluorenylmethoxy carbonyl-based solid-phase methods (32) in an Abimed 422 multiple synthesizer, as previously described (31). After trifluoroacetic acid cleavage, the crude materials were analyzed for identity by MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectroscopy and for purity by reversed-phase high-pressure liquid chromatography (HPLC). They were found to be correct by both criteria. Any sequences that failed to give the expected mass and/or that showed <75% purity by HPLC were resynthesized under carefully controlled conditions (manual assembly, ninhydrin monitoring, recoupling as required) until the above requirements were met.

In addition, two tandem peptides containing the sequence of the continuous B-cell epitope VP1[137–156] juxtaposed to the T-cell epitope 3A[21–35], in the two possible orientations (peptides BT and TB; Table 1), were prepared. Both peptides were synthesized as C-terminal carboxamides by Boc-based solid-phase methods (12) in semiautomated mode, with systematic ninhydrin control and recoupling as required. Preliminary deprotection-cleavage experiments (HF–*p*-cresol, 9:1, 0°C, 1 h) showed substantial oxidation to sulfoxide of the Met residue in each sequence. In order to minimize this side reaction, acidolysis of the peptide resins was conducted under low-high hydrogen fluoride conditions (52). Even so, crude complexes were obtained in both cases, requiring two consecutive reversed-phase HPLC purification steps to give fairly homogeneous (>90%) products, with correct amino acid analysis and MALDI-TOF mass spectral data. B-cell antigenicity of these tandem peptides was confirmed as they reacted in dot blot assays against MAbs that recognized the peptide VP1[137–156] (7).

Lymphoproliferation assays. Proliferation assays with porcine lymphocytes were performed as described elsewhere (39). Blood was collected in 5 μM EDTA and used immediately for the isolation of peripheral blood mononuclear cells (PBMC) (45). Assays were performed in 96-well round-bottom microtiter plates (Nunc). Briefly, 2.5×10^5 PBMC per well were cultured in triplicate, in a final volume of 200 μl, in complete RPMI, 10% (vol/vol) fetal bovine serum, and 50 μM 2-mercaptoethanol in the presence of various concentrations (fivefold dilutions) of the following: FMDV, ranging from 4×10^3 to 2.5×10^6 PFU/ml; nitrocellulose-bound viral polypeptides, ranging from 0.02 to 2.5 μg/ml; and synthetic peptides, ranging from 0.8 to 100 μg/ml. Control cultures without viral antigens were included. Cells were incubated at 37°C in 5% CO_2 for 4 days. After incubation, each well was pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine for 18 h. The cells were collected using a cell harvester, and the incorporation of radioactivity into the DNA was measured by liquid scintillation in a Microbeta counter (Pharmacia). Results were expressed as stimulation indexes (SI) (mean counts per minute [cpm] of stimulated cultures/mean cpm of control cultures). Lymphoproliferations that induced SI of ≥3 were considered as positive.

For the analysis of the role played by the MHC in the lymphoproliferations, the following murine anti-swine MHC swine leukocyte antigen (SLA) MAbs were used: 74-11-10 (immunoglobulin G [IgG2b]) anti-SLA class I (35) and MSA-3 (IgG2a) anti-SLA class II (28). The procedure was as described previously (7). A total of 15 μl of the appropriate MAb (1 mg/ml) was added per well of PBMC, stimulated with peptides as described above, at the beginning of culture, and an additional 15 μl per well was added after 24 h. Finally, the plates were processed as described above. These concentrations had been identified to give maximum specific blocking of SLA-restricted responses and have been successfully employed to such ends (7). The anti-MHC class II MAb concentrations have been shown to inhibit concanavalin A-, but not phorbol esters plus PMA ionophore (PMA)-, induced proliferation of swine PBMC, while the anti-MHC class I MAb did not affect these proliferations (11). Under the experimental conditions used, FMDV peptide-induced lymphoproliferations were not significantly inhibited by MAbs against the SWC3 monocytic marker and the γδTCR which acted as relevant negative controls (7).

Detection of FMDV neutralizing activity in supernatants of immune PBMC stimulated with peptides. About 10^6 PBMC from reinfected pigs were in vitro stimulated, as described above, with 20 μg of the different peptides per ml. PBMC from infected animals incubated in the presence of medium alone were used as controls. Culture supernatants were collected after 4, 7, and 13 days of stimulation, and the FMDV neutralizing activity was analyzed using a modification of the PFU reduction assay previously described (40). Briefly, ca. 60 to 80 FMDV PFU were incubated for 45 min, with or without 125 μl of the supernatants. The mixtures were employed to infect BHK-21 cell monolayers (ca. 10^6 cells), and the infection was allowed to proceed for 18 h in the presence of low-melting-point agarose (1.3%). The monolayers were fixed and stained with 10% (vol/vol) acetic acid–0.5% (wt/vol) crystal violet, and virus PFU were scored. PFU reductions were expressed as the percentage of PFU observed in the presence of peptide-stimulated supernatants with respect to the PFU in the presence of supernatant from PBMC incubated with medium alone.

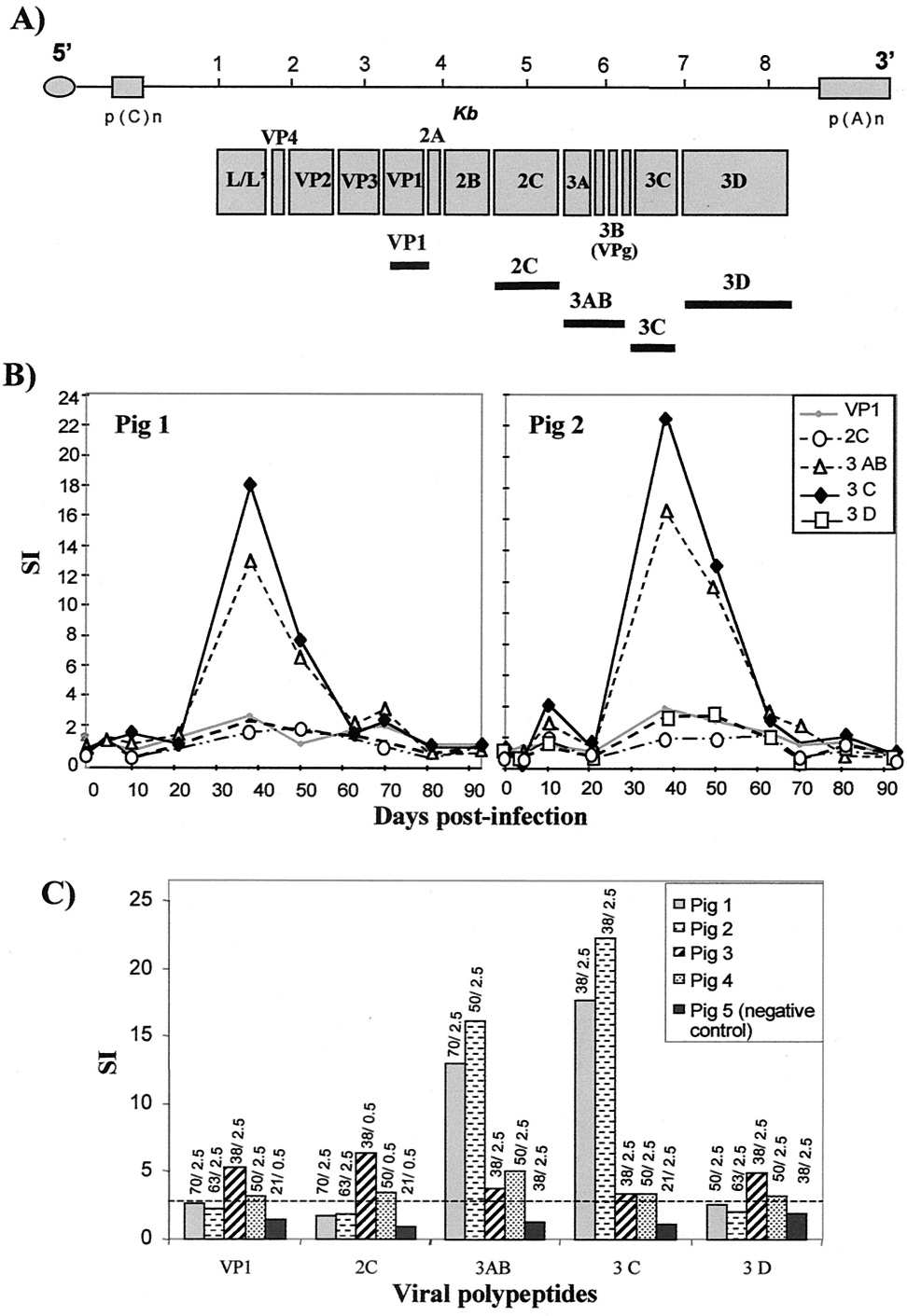

FIG. 1. Heterotypic lymphoproliferative response to viral polypeptides by FMDV-infected pigs. Fusion polypeptides from type O isolate (O1Kb) were used to in vitro stimulate PBMC from pigs infected with type C isolate (C-S8), as described in Materials and Methods. (A) Schematic representation of FMDV genomic RNA showing the location in the viral polyprotein of the fusion polypeptides used in this study. (B) Time course study of the lymphoproliferative response to FMDV polypeptides by lymphocytes from infected pigs 1 and 2. Assays were performed with lymphocytes obtained at 0, 4, 10, 21, 38, 50, 63, 70, 81, and 93 d.p.i. In those d.p.i. (i.e., from 10 to 80) in which positive SI were observed, peak responses were obtained with a nitrocellulose-bound protein concentration of 2.5 μg/ml, with the following exceptions in which the highest stimulation was obtained at a concentration of 0.5 μg/ml: VP1, day 10 (pigs 1 and 2); 3ABC, day 50 and 63 (pig 1) and days 63, 70, and 80 (pig 2); 3C, day 63 (pig 2); and 3D, day 63 (pig 1). (C) Peak lymphoproliferative response to FMDV polypeptides by the four pigs (animals 1 to 4) analyzed. The dotted line represent the value (SI ≥3), above which responses were considered positive. The d.p.i. values, followed by the nitrocellulose-bound protein concentration (in micrograms per milliliter) corresponding to each of the responses, are shown above each bar (i.e., "70/2.5" indicates an SI obtained at 70 d.p.i. with 2.5 μg of nitrocellulose-bound protein per ml). In all cases, The results are expressed as SI as described in Materials and Methods. The standard deviations of these values never exceeded 15% of the mean. A control animal was inoculated with PBS. The background cpm values (obtained with lymphocytes incubated with medium alone) were 502 (pig 1), 293 (pig 2), 1,140 (pig 3), 971 (pig 4), and 508 (control pig 5).

TABLE 1. Synthetic peptides used in this study

Peptide[a]	No.	Amino acid sequence[b]	Residues[c]
3A	1	ISIPSQKSVLYFLIE	1–5
	2	QKSVLYFLIEKGQHE	6–20
	3	YFLIEKGQHEAAIEF	11–25
	4	KGQHEAAIEFFEGMV	16–30
	5	AAIEFFEGMVHDSIK	21–35
	6	FEGMVHDSIKEELRP	26–40
	7	HDSIKEELRPLIQQT	31–45
	8	EELRPLIQQTSFVKR	36–50
	9	LIQQTSFVKRAFKRL	41–55
	10	SFVKRAFKRLKENFE	46–60
	11	AFKRLKENFEIVALC	51–65
	12	KENFEIVALCLTLLA	56–70
	13	IVALCLTLLANIVIM	61–75
	14	LTLLANIVIMIRETH	66–80
	15	NIVIMIRETHKRQKM	71–85
	16	IRETHKRQKMVDDAV	76–90
	17	KRQKMVDDAVNEYIE	81–95
	18	VDDAVNEYIEKANIT	86–100
	19	NEYIEKANITTDDQT	91–105
	20	KANITTDDQTLDEAE	96–110
	21	TTDDQTLDEAEKNPLE	101–115
	22	LDEAEKNPLETSGAS	106–120
	23	KNPLETSGASTVGFR	111–125
	24	TSGASTVGFRERTLP	116–130
	25	TVGFRERTLPGQKACD	121–135
	26	ERTLPGQKACDDVNS	126–140
	27	GQKACDDVNSEPAQP	131–145
	28	DDVNSEPAQPTEEQP	136–150
	29	EPAQPTEEQPQAEGP	141–155
	30	TEEQPQAEGPYAGPL	146–153
3B	1	QAEGPYAGPLERQRP	1–12
	2	YAGPLERQRP LKVRA	3–17
	3	ERQRP LKVRAKLPRQ	8–22
	4	LKVRAKLPRQE	13–23
	5	GPYAGPMERQKPLKV	24–38
	6	PMERQKPLKVKARAP	29–43
	7	KPLKVKARAPVVKEG	34–48
	8	KARAPVVKEGPYEGP	39–53
	9	VVKEGPYEGPVKKPV	44–58
	10	PYEGPVKKPVALKVK	49–63
	11	VKKPVALKVKAKNLI	54–68
	12	ALKVKAKNLIVTE	59–73
3C	1	SGAPPTDLQKMVMGN	1–15
	2	TDLQKMVMGNTKPVE	6–20
	3	MVMGNTKPVELILDG	11–25
	4	TKPVELILDGKTVAI	16–30
	5	LILDGKTVAICCATG	21–35
	6	KTVAICCATGVFGTA	26–40
	7	CCATGVFGTAYLVPR	31–45
	8	VFGTAYLVPRHLFAE	36–50
	9	YLVPRHLFAEKYDKI	41–55
	10	HLFAEKYDKIMLDGR	46–60
	11	YDKIMLDGRALTDS	51–65
	12	MLDGRALTDSDYRVF	56–70
	13	ALTDSDYRVFEFEIK	61–75
	14	DYRVFEFEIKVKGQD	66–80
	15	EFEIKVKGQDMLSDA	71–85
	16	VKGQDMLSDAALMVL	76–90
	17	MLSDAALMVLHRGNR	81–95
	18	ALMVLHRGNRVRDIT	86–100
	19	HRGNRVRDITKHFRD	91–105
	20	VRDITKHFRDVARMK	96–110
	21	KHFRDVARMKKGTPV	101–115
	22	VARMKKGTPVVGVIN	106–120
	23	KGTPVVGVINNADVG	111–125
	24	VGVINNADVGRLIFS	116–130
	25	NADVGRLIFSGEALT	121–135
	26	RLIFSGEALTYKDIV	126–140
	27	GEALTYKDIVVCMDG	131–145
	28	YKDIVVCMDGDTMPG	136–150
	29	VCMDGDTMPGLFAYR	141–155
	30	DTMPGLFAYRAATKA	146–160
	31	LFAYRAATKAGYCGG	151–165
	32	AATKAGYCGGAVLAK	156–170
	33	GYCGGAVLAKDGADT	161–175
	34	AVLAKDGADTFIVGT	166–180
	35	DGADTFIVGTHSAGG	171–185
	36	FIVGTHSAGGNGVGY	176–190
	37	HSAGGNGVGYCSCVS	181–195
	38	NGVGYCSCVSRSML	186–200
	39	YCSCVSRSMLLKMKA	191–205
	40	RSMLLKMKAHIDPEP	196–210
	41	KMKAHIDPEPHHE	201–215
BT_{3A-5}[d]		TASARGDLAHLTTTHARH LP*AAIEFFEGMVHDSIK*	137–156[e]
$T_{3A-5}B$[d]		*AAIEFFEGMVHDSIK*TASAR GDLAHLTTTHARHLP	21–35[f]

[a] Peptides were 15 amino acids in size and overlapped by 10 amino acids. The viral NSP corresponding to each set of peptides (3A, 3B, and 3C) is indicated.
[b] Peptide amino acid sequence as in reference (51).
[c] Amino acid positions spanned by each peptide in the corresponding protein. The carboxy-terminal residue of each NSP is underlined. Peptide 3A-30 included the six amino-terminal residues of 3B. Peptide 3B-1 included the three carboxy-terminal residues of 3A. The sequences corresponding to VP1 and 3A, including in the tandem peptides, are shown.
[d] Tandem peptides, in which the VP1 and 3A residues are indicated, were colinearly synthesized in the two possible orientations (BT and TB). The 3A sequences are in italics.
[e] That is, VP1 [137–156].
[f] That is, 3A [21–35].

RESULTS

Lymphoproliferative responses to FMDV NSP. In order to study the contribution of the NSP to the T-cell response elicited by FMDV in swine, type O polypeptides expressed in *E. coli* (Fig. 1A) were used to stimulate in vitro PBMC from four outbred pigs (animals 1 to 4) infected with type C isolate (C-S8). The experimental approach was designed to identify regions conserved among different FMDV serotypes, which were capable of inducing heterotypic T-cell responses in lymphocytes from domestic pigs. Lymphoproliferative responses against the different polypeptides were monitored using PBMC obtained from 0 to 100 days p.i. (d.p.i.). The magnitude of the responses observed showed animal-to-animal variation, but a consistent trend was noted. Positive lymphoproliferations (SI $\geq$3) against NSP and VP1 capsid protein (also included in this analysis) were detected from day 38 p.i. until day 70 p.i., and the higher SI were observed at between 38 and 50 d.p.i. No stimulations were observed with PBMC obtained from the animals prior to infection (day 0 p.i.) nor from a control animal inoculated with PBS (pig 5). Figure 1B shows the results found with cells from the highest-responder pigs 1 and 2, which were representative of the results obtained with cells from the four

TABLE 2. Peptides corresponding to 3A, 3B, and 3C that were significantly recognized by lymphocytes from FMDV-infected pigs

Peptide[a]	No.	Amino acid residues[b]	SI[c] for pig:					
			6	7	8	9	10	11
3A	3	11–25	ND	5.3*	14.7	8.1*	18.2	2.5*
	5	21–35	16.4	7.3*	20.2	3.2*	4.2	4.6†
	6	26–40	7.6*	5.8	18.5	3.6*	14*	2.4†
	9	41–55	7.3	9.7	2.8	4†	6.6	4.8*
	25	121–135	ND	7.7	8.1	2.9	3.5	6.2†
3B	4	13–23	2.5	10.8	3.5	5.6*	5.2	2.2*
	8	39–53	3.3	11.6	2.1	7.3†	5.5	4.3*
3C	11	51–65	ND	17.2	12.6	6.7	10.4	2.7
	12	56–70	6.1*	18.6	22.4	6	4.5	5.4
	13	61–75	7.1	7.8	19.7*	6.2*	2.8	7.1*
	19	91–105	3†	5.5†	15.2*	6.6*	7.4	2.9*
	20	96–110	2†	18.1*	2.5*	4.5*	3.6	3.9
	25	121–135	9.8	16.6	14	4.3	7.7	2.5†
	27	131–145	10.8*	27.4	17.3	7.3	3.2	9.2*
	28	136–150	ND	14.8	10.6	3.2	4	2.7*
	33	161–175	ND	15.6	4.5	3.9	6	2.9*
	34	166–180	ND	16.6	18.2	4.1	15	4.4†
	39	191–205	ND	5.4	2.5	7	3.5	4.2†
	40	196–210	2.2	20.9	3.7	6.8	7.3	7.3
C-S8[d]			24.3	24.7	ND	10.7	19	ND

[a] Peptides that induced an SI of >3 in at least four of the six pigs analyzed. The corresponding protein to which the peptides belong is indicated.

[b] Amino acid residues spanned by each of the peptides in the 3A, 3B, or 3C NSP.

[c] That is, the highest SI induced by the different antigens in PBMC isolated at day 28 p.i. Unless indicated otherwise, optimal responses were obtained with a peptide concentration of 100 μg/ml. ND, not done. *, SI was obtained with a peptide concentration of 20 μg/ml; †, SI was obtained with a peptide concentration of 4 μg/ml.

[d] SI induced by C-S8 virus. The optimal responses shown were obtained with a viral concentration of 5×10^5 PFU/ml.

animals analyzed. In general, the responses were dose dependent, and the higher SI were obtained with nitrocellulose-bound protein at from 0.5 to 2.5 μg/ml. Figure 1C shows the peak responses found in each of the animals analyzed. Polypeptides 3AB and 3C were recognized by the lymphocytes from all the animals analyzed. Proteins 2C, 3D, and VP1 were recognized by cells from only two of the four pigs. These results suggest the presence within the 3ABC polypeptide region of T-cell epitopes conserved among FMDV serotypes, which are consistently recognized by lymphocytes from domestic pigs.

Identification of T-cell epitopes in 3A, 3B, and 3C. In order to characterize further the T-cell epitopes located in the 3ABC region, a set of overlapping peptides (15-mer), covering proteins 3A, 3B, and 3C of the C-S8 virus, was synthesized. These peptides were employed to stimulate in vitro lymphocytes from infected pigs (see Materials and Methods and Table 1 for details). For this purpose, six additional pigs (animals 6 to 11) were experimentally infected with C-S8 virus, and their PBMC were isolated on days 14 and 28 p.i. None of the peptides induced positive SI in PBMC obtained from these animals before infection (data not shown). In general, the responses against individual peptides were already detectable at day 14 p.i., being more consistent at day 28 p.i. They were dose dependent, and the highest values were obtained with peptide concentrations ranging from 4 to 100 μg/ml (Table 2). Among the 83 overlapping peptides tested, 45 did not induce significant proliferations in any of the pigs analyzed. The peak responses against the 19 peptides that induced positive SI in at least four of the six animals are indicated in Table 2. The magnitude of the response against individual peptides correlated with the SI observed against the whole virus, but the pattern of peptide recognition was different in each of the animals studied. However, three individual peptides consistently stimulated PBMC from the six pigs analyzed: 3A-5[21–35], 3C-12[56–70], and 3C-27[131–145] (see Table 2).

For studies on the T-cell antigenic specificity elicited upon challenge, animals 7 to 11 were reinoculated 4 months after the initial infection with a heterologous type O FMDV virus (O-BFS). The five pigs developed an acute episode of the disease, and PBMC obtained at days 10, 21, and 35 postreinfection (p.r.i.) were used to perform lymphoproliferative assays in which the 3ABC peptides were used as stimulator antigens. In general, the responses against peptides were detected at day 21 p.r.i., being more consistent at day 35 p.r.i. The responses were dose dependent, and the highest values were obtained with peptide concentrations (4 to 100 μg/ml) similar to those found with cells after the first infection (Table 3). In this experiment, positive SI were also induced with peptide concentrations lower than those effective with cells from the initially infected animals (0.8 μg/ml; data not shown). The efficacy of the heterologous restimulation showed individual variation among the animals. A clear boost of individual peptide responses was observed for lymphocytes from animal 8. Lymphocytes from animals 9, 10, and 11 showed an increased, albeit of

TABLE 3. Peptides corresponding to 3A, 3B, and 3C, that were significantly recognized by lymphocytes from FMDV-reinfected pigs

Peptide[a]	No.	Amino acid residues[b]	SI[c] for pig:				
			7	8	9	10	11
3A	3[d]	11–25	3.2	20	4.2	28.6*	2.1*
	5[d]	21–35	5.7	63.4	5.9*	10.6	3.3†
	6[d]	26–40	2.6*	40.2	5*	8.5*	2.9*
	9[d]	41–55	4.1*	13.4*	7†	6.4	1.9†
	17	81–95	4.5	29.7*	4.4	19.3	1.4*
3B	2	3–17	5.5	10.1	8.4	3.5*	1.1*
	4[d]	13–23	2.1	9.6	8	8.1	2.6*
3C	12[d]	56–70	4.1	93.4	17.3	5.2	9.2
	13[d]	61–75	4.1	98.8*	13.1†	5.2	9.2*
	20[d]	96–110	2.7	99.5	1.8*	5.4	8.7
	25[d]	121–135	3.1	42.9*	3.5	7.1	ND
	26	126–140	15.9	10.5*	4.9	4.9*	ND
	30	146–160	7.6	102.2	ND	6.5	ND
	32	156–170	6.7	63.9	ND	5.8	ND
	34[d]	166–180	10.2	42	2.8	33.2	10.9†
	40[d]	196–210	8.6	10.8	8.9	4.2	11.5
C-S8[e]			107.5	307.9	26	56.2	ND
O-BFS[e]			217.3	112.4	11	52.6	ND

[a] Peptides that induced an SI of > 3 in at least three of the five pigs analyzed.

[b] Amino acid residues spanned by each peptides in the 3A, 3B, or 3C NSP.

[c] That is, the highest SI induced by the different antigens in PBMC isolated at day 35 p.r.i. Unless indicated otherwise, optimal responses were obtained with a peptide concentration of 100 μg/ml. ND, not done. *, SI obtained with a peptide concentration of 20 μg/ml; †, SI obtained with a peptide concentration of 4 μg/ml.

[d] Peptides that induced positive SI in at least four of the six initially infected pigs (see Table 2).

[e] SI induced by C-S8 or O-BFS viruses. The optimal responses shown were obtained with a viral concentration of 5×10^5 PFU/ml.

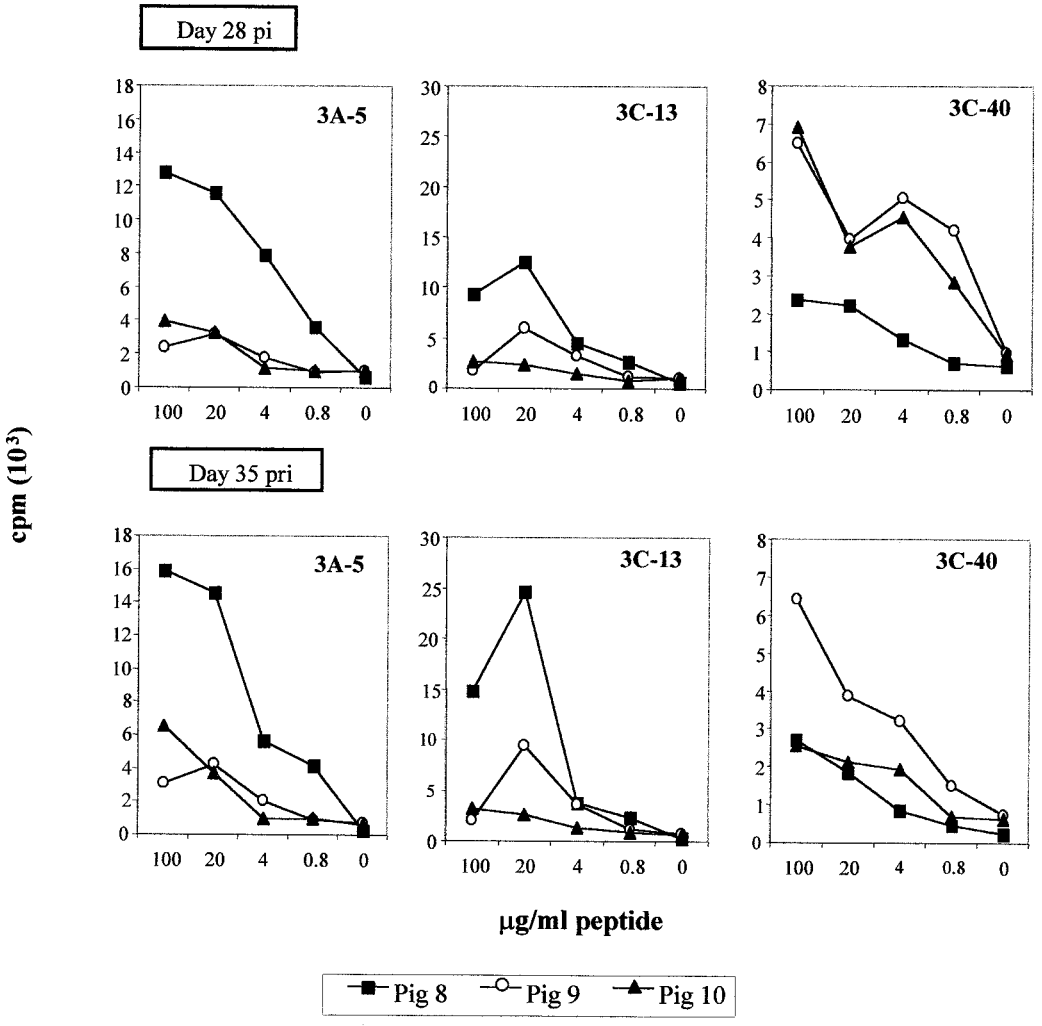

FIG. 2. Effect of peptide dose on the proliferative response of PBMC obtained from pigs 8, 9, and 10 at days 28 p.i. and 35 p.r.i. The values corresponding to 0 on the *x* axis indicate the background of the assay (PBMC incubated with medium alone).

lower magnitude, in most of the SI, and the responses of PBMC from animal 7 were lower than those observed after the first infection. Table 3 summarizes the SI obtained with the peptides that induced positive responses in at least three of the five animals analyzed.

Lymphocytes from reinfected pigs responded to 11 of the 19 peptides identified as antigenic after analyses with cells obtained following the first infection (Table 3). Peptides 3A-3, -5, -6, and -9, peptide 3B-4, and peptides 3C-12, -13, -20, -25, -34, and -40, previously identified as good stimulators (see Table 2), also induced positive responses in at least three of the five reinfected pigs. In addition, the following new peptides induced proliferation in lymphocytes taken from the majority of the reinfected animals analyzed: 3A-17; 3B-2; and 3C-26, -30, and -32. Again, several individual peptides induced lymphoproliferative responses by cells from the five animals upon heterologous reinfection (Table 3): 3A-5[21–35], 3C-12[56–70], 3C-13[61–75], 3C-34[166–180], and 3C-40[196–210]. Representative dose responses induced by some of these peptides in PBMC from infected and reinfected animals are shown in Fig. 2.

SLA restriction of the anti-peptide response. Information about the SLA restriction of the detected lymphoproliferative responses was obtained using MAbs against SLA class I and class II. These were used to inhibit in vitro the lymphoproliferations induced in cells from reinfected pigs. The peptides 3A-3[11–25], 3A-5[21–35], 3C-34[166–180], and 3C-40[196–210] were selected for this study because they induced consistent responses in cells from most of the analyzed animals, after both the first infection and the heterologous re infection (Tables 2 and 3). Results showed the highest inhibition of peptide-induced lymphoproliferation (>75%) when the anti-SLA class II MAb was used. This is consistent with lymphoproliferations induced by peptides being dependent on $CD4^+$ T -helper lymphocyte activities (Table 4). The anti-SLA class I MAb was also inhibitory, but the magnitude of inhibition was generally lower than that observed with the anti-SLA class II antibody.

Antigenicity of tandem peptides including B- and T-cell epitopes. The potential of the T-cell epitopes identified in NSP to improve vaccine formulation of FMDV synthetic peptides requires that T-cell epitopes remain immunostimulatory when in combination with B-cell epitopes. In order to investigate this

TABLE 4. Inhibition by MAb to porcine class I and class II of the proliferative response to 3A and 3C peptides

Peptide[a]	MAb[b]	Inhibition in pig[c]: 9 Δcpm	9 %	8 Δcpm	8 %
3A-3[11–25]	None	8,615 ± 980		4,101 ± 615	
	Anti-class I	2,249 ± 337	73.8	2,740 ± 411	33.1
	Anti-class II	2,850 ± 484	66.9	905 ± 108	77.9
3A-5[21–35]	None	9,599 ± 1151		7,853 ± 471	
	Anti-class I	4,007 ± 640	58.2	3,807 ± 609	51.2
	Anti-class II	1,938 ± 193	79.8	1,525 ± 213	59.9
3A-34[166–180]	None	17,389 ± 869		9,808 ± 689	
	Anti-class I	5,692 ± 1024	67.2	3,757 ± 676	61.6
	Anti-class II	2,701 ± 243	84.4	2,484 ± 298	74.6
3C-40[196–210]	None	7,360, ± 1030		5,292 ± 793	
	Anti-class I	2,854, ± 342	61.2	4,626 ± 277	12.5
	Anti-class II	1,145 ± 171	84.4	2,210 ± 198	58.2

[a] The characteristics of the peptides are described in Table 1. Peptides were used at a concentration of 20 μg/ml. None, lymphocytes cultured in the absence of MAb.

[b] A total of 15 μl (1 mg/ml) of MAb anti-SLA class I (74-11-10, Ig-G2b) or class II (MSA-3, IgG2a) was added per well at the beginning of culture. After 24 h, the cultures were supplemented with another 15 μl of MAb.

[c] Lymphocytes from pigs 8 and 9, obtained at day 35 p.r.i. were used in these experiments.

point, the immunostimulatory potential of the T-cell peptide 3A-5[21–35] was further analyzed by using tandem peptides in which this epitope was colinearly synthesized with the B-cell site VP1[137–156]. Two possible orientations were used: peptides BT and TB (Table 1). With lymphocytes from pigs 7, 9, 10, and 11, the tandem epitopes induced lymphoproliferative responses in vitro upon infection with C-S8 virus, as shown in Fig. 3A. The responses were dose dependent, and the highest SI was obtained with a peptide concentration of 20 μg/ml. Positive SI were found with cells from all four animals stimulated with peptide $T_{3A-5}B$, whereas only cells from animals 7 and 11 responded to peptide BT_{3A-5}. The SI of the latter cells were lower than those induced by peptide $T_{3A-5}B$. In this experiment, peptide 3A-5[21–35] was also recognized with an SI lower than those found using peptide $T_{3A-5}B$.

Upon reinfection with the heterologous O-BFS virus, lymphocytes from pigs 7, 8, and 10 responded to $T_{3A-5}B$, again with SI higher than those induced by peptide 3A-5[21–35] alone (Fig. 3B). Thus, tandem peptide $T_{3A-5}B$ stimulates in vitro lymphocytes from both infected and reinfected animals.

It was then interesting to address whether the incorporation of 3A-5[21–35] into the tandem peptide could result in the stimulation of T cells to provide the necessary immunological help for antigen-stimulated B lymphocytes. To this end, the stimulation of T cells by peptide $T_{3A-5}B$ was analyzed in terms of cooperation with the induction of an antiviral humoral response. Lymphocytes from reinfected pig 8 were stimulated in vitro with peptides $T_{3A-5}B$, T, or B. At different times poststimulation, the presence of anti-FMDV activity in the supernatants was measured using a plaque reduction assay. Significant PFU reductions were detected using supernatants from cultures stimulated with peptide $T_{3A-5}B$ from the fourth day, reaching a maximum with day 7 supernatants (ca90%) (Fig. 4). The PFU reductions were higher than those obtained using supernatants from cultures stimulated by peptides T and B separately. In addition, the antiviral activity was restricted to the homologous virus C-S8, since no plaque reduction was detected when a type O FMDV was used in the assay (Fig. 4). Thus, a serotype-specific anti-FMDV activity, consistent with the induction of anti-FMDV neutralizing antibodies, was detected upon stimulation of immune lymphocytes with peptide $T_{3A-5}B$.

DISCUSSION

In the present studies the antigenic specificity of the porcine (an important natural host of this virus) T-cell response against FMDV NSP was analyzed. The aim was the identification of T-cell epitopes in NSP widely recognized by domestic pigs, the

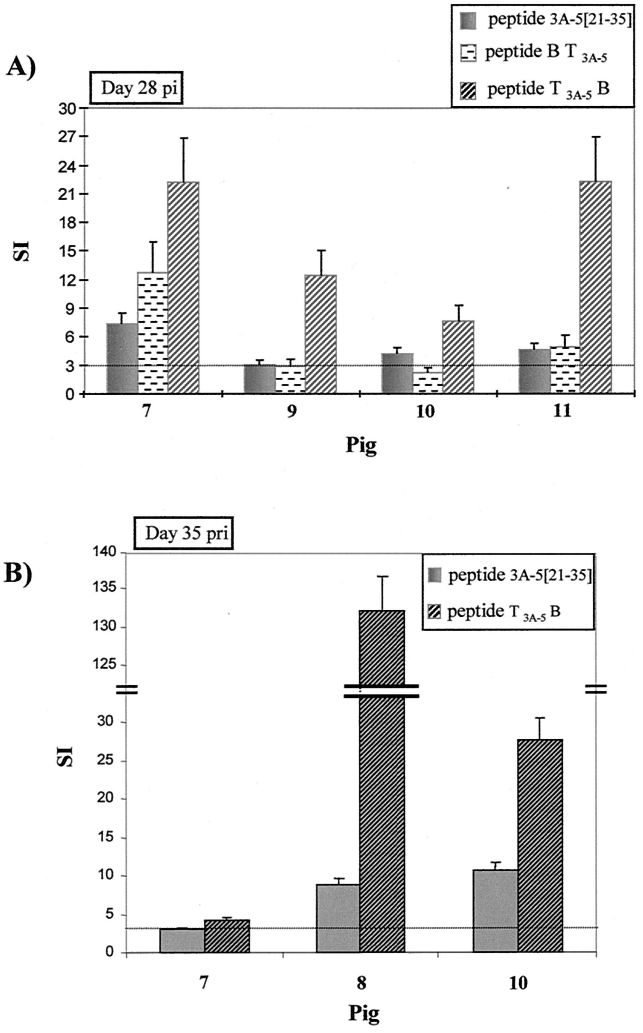

FIG. 3. Lymphoproliferative responses to peptides 3A-5[21–35], BT_{3A-5}, and $T_{3A-5}B$. The data shown correspond to peak responses of lymphocytes obtained at days 28 p.i. with C-S8 virus (A) and 21 p.r.i. with the heterologous O-BFS virus (B). The standard deviations are indicated. The peptide concentrations giving the SI shown were 20 μg/ml, except for pig 9 (in panel A) in which the peak response was obtained with 100 μg/ml. The background cpm levels were 405 (pig 7), 450 (pig 9), 525 (pig 10), and 345 (pig 11) (A) and 302 (pig 7), 214 (pig 8), and 258 (pig 10) (B).

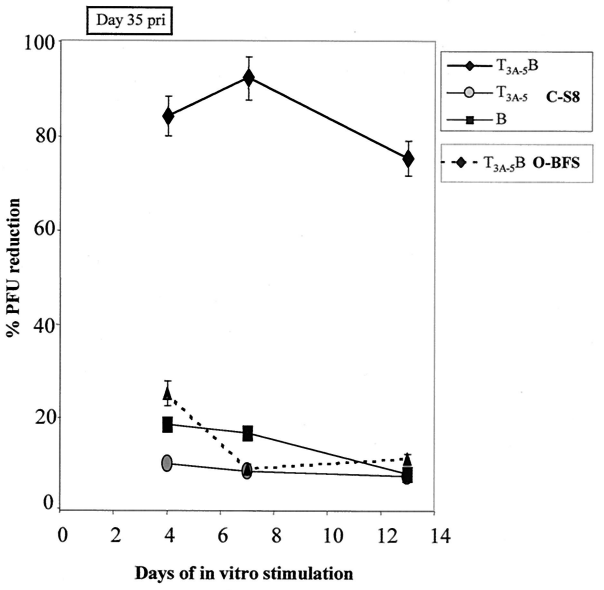

FIG. 4. FMDV PFU reduction by supernatants of immune PBMC stimulated in vitro with viral peptides. The data correspond to PBMC from pig 8 (obtained at day 35 p.r.i.) assayed at different days of in vitro stimulation with 20 μg of the indicated peptides per ml. The results are expressed as the percent inhibition in the PFU recovered from cells stimulated with each peptide with respect to the PFU observed with a control supernatant incubated with medium alone. The assay was performed using two FMDV isolates of different serotypes: C-S8 (type C) and O-BFS (type O). The standard deviations are indicated.

sequences of which were conserved among different FMDV serotypes. Animals were selected from different litters to ensure a representative sample of outbred pigs and to bring porcine MHC polymorphism in the T-cell epitope recognition into the study. FMDV NSP are absent, or incorporated in very small amounts, in the virus particle (34). Therefore, the analyses used animals experimentally infected with a type C FMDV isolate.

T-cell antigenicity of the NSP was first analyzed in terms of the capacity of different polypeptides to stimulate in vitro T cells from the infected animals. A heterotypic lymphoproliferative response against NSP in cells from FMDV-infected pigs was tested. Lymphoproliferations were detected from day 38 to day 70 p.i. Although the SI showed animal-to-animal variations, polypeptides 3AB and 3C induced specific and consistent responses in lymphocytes from all animals analyzed. Polypeptides 2B and 3D and capsid protein VP1 were not recognized by all the animals (Fig. 1C). This finding contrasts with the recognition of type O FMDV 3D as an immunodominant T-cell determinant by cattle lymphocytes, as recently reported (16). In addition to differences between cattle and swine in terms of antigenic recognition patterns, the lower antigenicity of 3D for porcine lymphocytes may also reflect differences between FMDV type C and type O. The low heterotypic recognition of VP1 is consistent with the sequence divergence that exists between type O and type C FMDV.

Overlapping synthetic peptides allowed a detailed analysis of the T-cell epitopes recognized in proteins 3A, 3B, and 3C. Despite animal to animal variation, 19 peptides were stimulated in vitro lymphocytes from, at least, four of the six infected pigs analyzed (Table 2). The responsive peptides define T-cell regions efficiently recognized by swine lymphocytes at the following amino acid positions: 3A[11–55] and -[121–135], 3B[13–23] and -[39–53], and 3C[51–75],-[91– 110], -[121–150], -[161–180], and -[191–210]. Eleven of these peptides were also recognized by lymphocytes from at least three of the five heterotypically reinfected pigs analyzed (Table 3). Five new peptides, which did not induced in vitro responses in the initially infected animals, became responsive upon the induction of a secondary response (Table 3). The efficacy of the heterologous restimulation was uneven among the five animals studied. A clear boost of individual peptide responses was only observed for lymphocytes from animal 8. 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 a number of peptide epitopes in the nonstructural polypeptides 3A, 3B, and 3C of FMDV, which are capable of stimulating porcine T cells following infection with viruses of different serotypes, and that some of them may boost primed lymphocytes.

SLA class II MAb efficiently inhibited the lymphoproliferative response. This demonstrated the involvement of $CD4^+$ Th lymphocytes in the recognition of some of the stimulatory peptides (Table 4). Anti-class I MAb gave lower inhibitions, in agreement with the previous reports on porcine lymphoproliferation against whole virus (14, 45) and FMDV capsid protein peptides (7). These inhibitions might relate to the high proportion of $CD8^+$ $CD4^+$ double-positive cells found in swine (58), dominated by memory Th lymphocytes (50, 59). Certainly, it must also be considered that the animals were infected with FMDV and, therefore, that stimulation of $CD8^+$ cells through class I presentation could have occurred (14). The implication of $CD8^+$ lymphocytes in protective responses to FMDV remains largely unknown and thus its characterization was not pursued here. Attempts to obtain porcine T-cell clones, a highly valuable tool for understanding the antigenic specificity and function of T cells, have had very limited success. Thus, as a first step in the characterization of the immune responses induced by the T-cell epitopes thus for identified, our attention was focused on the potential of these peptides to stimulate $CD4^+$ cells to provide T help to immune B lymphocytes. In this way, we attempted to identify T-cell epitopes that could enhance the induction of anti-FMDV antibodies by peptide vacines. For practical reasons, such T-cell epitopes should be conserved among different FMDV serotypes. Peptides 3A-3[11–25], 3A-5[21–35], 3C-25[121–135], and 3C-34[166–180] do indeed show amino acid sequences completely conserved among serotypes A, O, and C (Table 5). These 3A and 3C peptides are frequently and efficiently recognized by porcine lymphocytes in both a homotypic and heterotypic manner. Consequently, they constitute potential candidates for inclusion in a peptide vaccine formulation.

The colinear expression of T-helper and B-cell epitopes in peptide vaccines can result in the enhancement of antibody responses (8, 17). The design of functional combinations of T-cell and B-cell epitopes is rather empirical (19, 47) and should maintain the immunostimulatory ability of the B- and T-cell components. T-cell peptide 3A-5[21–35] consistently stimulated lymphocytes from all initially infected and reinfected animals analyzed in this study, as well as lymphocytes

TABLE 5. Alignment of the amino acid sequences corresponding to NSP 3A and 3C

Protein	Virus	Sequence[a]
3A	C-S8	ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKR 50
	O-BFS	
	A12	
	C-S8	AFKRLKENFEIVALCLTLLANIVIMIRETHKRQKMVDDAVNEYIEKANIT 100
	O-BFS	R
	A12	R
	C-S8	TDDQTLDEAEKNPLETSGASTVGFRERTLPGQKARDDVNSEPAQPTEEQP 150
	O-BFS	K S C V
	A12	T T R CN R A
	C-S8	QAE 153
	O-BFS	
	A12	
3C	C-S8	SGAPPTDLQKMVMGNTKPVELILDGKTVAICCATGVFGTAYLVPRHLFAE 50
	O-BFS	
	A12	
	C-S8	KYDKIMLDGRALTDSDYRVFEFEIKVKRGQDMLSDAALMVLHRGNRVRDI 100
	O-BFS	V M
	A12	M
	C-S8	TKHFRDVARMKKGTPVVGVINNADVGRLIFSGEALTYKDIVVCMDGDTMP 150
	O-BFS	T
	A12	T V
	C-S8	GLFAYKAATKAGYCGGAVLAKDGADTFIVGTHSAGGNGVGYCSCVSRSML 200
	O-BFS	R
	A12	S K
	C-S8	LKMKAHIDPEPHHE 214
	O-BFS	
	A12	R V Q

[a] Amino acid substitutions in FMDV of serotypes O (O-BFS) and A (A12) with respect to the C-S8 sequence are indicated. Sequences were obtained from the following Swiss-Prot accession numbers: Q9QCE3 (C-S8), POLG_FMDVA(A12), and POLG_FMDVO (O-BFS). Overdots indicate 10-amino-acid segments; numbers in italics indicate the cumulative amino acid count.

from four additional FMDV-infected domestic pigs (unpublished results). Therefore, peptide 3A-5[21–35] was employed to explore the potential of tandem peptides including the immunodominant B-cell site VP1[137–156], with either the BT or the TB orientation, to stimulate lymphocytes from infected animals. We first analyzed whether these tandem peptides retained the capacity to induce lymphoproliferation of immune lymphocytes. Each of the tandem peptides was recognized by immune lymphocytes, with $T_{3A\text{-}5}B$ being the most efficient stimulator of lymphocytes from both initially infected and reinfected animals (Table 3). Different factors may mediate the higher stimulations observed with tandem peptide $T_{3A\text{-}5}B$: among these factors is the influence of the different flanking sequences on the binding to MHC molecules that could modify lymphocyte activation (56) or on the susceptibility of the T-cell epitopes to proteases in antigen processing (20).

The induction of FMDV neutralizing antibodies by immune PBMC in response to in vitro viral stimulation has been previously reported as an indicator of T-cell–B-cell cooperation (40). Interestingly, a significant FMDV PFU reduction activity (up to 90%) was detected in supernatants of PBMC upon incubation with peptide $T_{3A\text{-}5}B$ under conditions in which the inhibitions induced by supernatants of PBMC stimulated with peptide A[21–35] or 3A-5[21–35] did not exceed 20%. This reduction was serotype specific, since it was not observed with a type O virus (Fig. 4). These results are consistent with the induction of anti-FMDV antibodies in the peptide-stimulated cultures, and new experiments are being conducted to further characterize the antiviral activity induced.

Inclusion of FMDV T-cell epitopes may enhance the immunogenicity of subunit and peptide vaccines. According to the model of intermolecular-intrastructural help (33, 43), B cells can be activated by T cells resulting from the stimulation by epitopes derived from a different protein, provided that both proteins are associated in a common complex. Whether 3ABC and viral structural proteins, VP1 in particular, may become associated in FMDV-infected cells remains to be studied. On the other hand, the stimulation of T cells by viral peptides may participate not only in providing T-cell help to B lymphocytes but also in eliciting a synergistic response against the virus infection. T cells secrete cytokines such as gamma interferon and interleukins that might play an important role in the pro-

tection against the virus (23, 57). Consistent with this hypothesis are the results described recently (46) that show that immunization with a recombinant adenovirus expressing the P1 polypeptide elicits partial protection against FMDV in pigs and cattle in the absence of antiviral antibody response. To explore these possibilities, experiments are in progress to assess the immunogenicity in pigs of combinations of the B-cell site VP1[140–160] and the T-cell peptides identified here.

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