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DNA immunization with 2C FMDV non-structural protein reveals the presence of an immunodominant CD8$^+$, CTL epitope for Balb/c mice

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Abstract

Outbreaks of foot and mouth disease virus (FMDV) have devastating economic consequences in affected areas. The presence of multiple serotypes and virus variants makes vaccination complicated. A better understanding of protective immune mechanisms may help in development of novel vaccines with cross protective capacity. While much attention has been devoted to humoral responses to FMDV, less is known about the role of cell-mediated responses in protective immunity. Predictions of potential CTL epitopes by two different computer algorithms identified the viral 2C protein as containing a potential murine H2-Kd CTL epitope located in its amino-terminal half. DNA vaccination of mice with a plasmid expressing the 2C protein and a fragment thereof confirmed that this was indeed a CTL epitope, as shown by interferon gamma (IFN-γ) induction in CD8$^+$, CD44^{hi} splenocytes after in vitro stimulation with peptides containing the amino acid sequence KYKDAKEWL, predicted for the CTL epitope. A peptide with the variant sequence KYKEAKEWL induced similar responses, indicating tolerability towards a conservative substitution at the altered residue. Virus infection likewise induced a measurable CTL response against KYKDAKEWL, although less clear due to a higher background of IFN-γ production in splenocytes from infected mice. Challenge of vaccinated mice showed that the CTL response induced by the 2C protein was not protective, since viremia and mortality were unaffected by vaccination. The implications for vaccine development are discussed in the context of cross-serotype reactive responses.

Keywords: Foot and mouth disease virus; Non-structural proteins; CTL epitope; DNA vaccination; Intracellular cytokine staining; Flow cytometry

1. Introduction

Foot and mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, and outbreaks have grave consequences for animal welfare and agricultural economy in affected areas. The disease is caused by foot and mouth disease virus (FMDV), a member of the family Picornaviridae, genus aphthovirus (reviewed by Bachrach, 1968). Outbreaks are controlled by strict measures targeting different disease transmission modes, including limitations on animal movement, killing of infected and contact herds and quarantine imposed on personnel and equipment, which has been in contact with infected herds. Due to the highly contagious nature of the virus, disease spread may be very difficult or impossible to avoid by these measures alone. Vaccination of herds may be an attractive supplementary action to be taken since it may reduce viral transmission both within and between herds (for a review, see Barnett and Carabin, 2002). If vaccination is to be applied as a control measure, its immediate implementation in areas surrounding outbreaks is required. FMDV is a very heterogenous virus, with seven serotypes and multiple subtypes (Domingo et al., 2003). The currently existing vaccines are based on inactivated virus and are serotype-specific and will thus not protect against heterologous viruses (Barteling and Vreeswijk, 1991; Doel, 2003). Since a forthcoming outbreak could be due to any serotype and subtype of virus, strategic reserves of several different types of vaccines are required at present (Barnett et al.,

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2001). Due to all these facts, development of new vaccines capable of inducing cross protection between serotypes is therefore attractive.

Protective immunity to FMDV is not yet fully understood. However, humoral response is known to be important, and vaccines based on inactivated antigens are highly effective in controlling disease and viral transmission. The neutralizing antibodies induced upon vaccination are highly serotype-specific, and thus do not confer protection against heterologous viruses (Barteling and Vreeswijk, 1991; Doel, 2003). Furthermore, in spite of vaccination with classical FMDV vaccines, development of persistent infection in ruminants (the so-called carrier animals) does occur [for a review, see Grubman and Baxt (2004) or Alexandersen et al. (2002)]. $CD4^+$ T-lymphocyte responses are induced both upon FMDV infection and vaccination, and there is evidence supporting their helper role in B-cell production of neutralising antibodies (Collen, 1994; McCullough and Sobrino, 2004; Sobrino et al., 2001). Also $CD8^+$ T-cell mediated immune responses have been reported to occur in immune pigs (Blanco et al., 2001; Garcia-Briones et al., 2004) and cattle (Childerstone et al., 1999). However, the significance of cell-mediated immune responses (CMI) in terms of protective immunity towards FMDV remains largely unknown, although some reports describe at least partial protection (Garcia-Briones et al., 2004; Sanz-Parra et al., 1999a). The tight interplay between innate immune responses, and FMDV specific T and B lymphocytes during viral infection in the natural hosts also complicates the interpretation of the relative importance of the individual effector mechanisms. In spite of these complications, several T helper epitopes have been described in both structural and non-structural FMDV proteins (Blanco et al., 2001; Collen et al., 1991; van Lierop et al., 1995; Zamorano et al., 1994), which may be included in new experimental vaccine formulations. Thus, vaccination of pigs with a combination of both FMDV B and T helper peptides induced detectable immune responses and conferred partial protection against virus challenge (Sanz-Parra et al., 1999a). DNA vaccines expressing B-cell sites in VP1 fused to an anti-host immunoglobulin conferred protection in mice in spite of limited levels of neutralizing antibodies (Wong et al., 2000). Also, co-expression of 3D polymerase with capsid proteins in a DNA vaccine has been reported to increase the protective responses conferred compared to capsid proteins alone (Cedillo-Barron et al., 2001). Thus, recent evidence from DNA immunization points towards a role of cellular immune responses in protection against FMDV infection.

Although T helper cell responses seem to play an important role in protection against FMD, the role of CTL responses has been less studied. In fact, to our knowledge, not a single CTL epitope has been fully characterized in FMDV. While studies of CTL responses in outbred populations are complicated, such studies are more straightforward in inbred mice. Balb/c mice are susceptible to infection with certain isolates of FMDV and may therefore be used as an animal model for FMDV infection (Fernandez et al., 1986; Francis et al., 1987; Salguero et al., 2005). Although the pathogenesis in mice and natural hosts differs, correlation has been observed from the immunological point of view. Protective mechanisms decribed as relevant in natural hosts (e.g. neutralizing antibodies), are also involved in protection against FMDV infection in mice. Indeed, neutralizing antibodies induced are mainly directed against the same epitopes in both mice and natural host animals (Crowther et al., 1993; Dunn et al., 1998). In this study, we investigated CTL responses against parts of the FMDV polyprotein which is well conserved among different serotypes. For this purpose, we DNA vaccinated Balb/c mice with a plasmid encoding the non-structural 2C protein of FMDV and tested for the post-vaccination and post-infection response in order to identify cross-reactive CTL epitopes. The 2C protein was chosen since it is one of the most well conserved proteins among serotypes (Carrillo et al., 2005), and this protein contains good candidate H2-d CTL epitopes as determined by two different epitope prediction algorithms. Finally, immune responses induced against essential early viral proteins, such as 2C (Grubman and Baxt, 2004), might help in controlling the initial spread of the virus.

The identification of conserved CTL epitopes will enable studies of the protective capacity of CTL responses in the mouse model of FMDV infection, and may provide clues on how to induce cross-protection between serotypes in the natural host animals.

2. Materials and methods

2.1. Virus and cells

The FMDV plaque isolate C-S8c1 (CISA-INIA, Valdeolmos, Spain), was used to inoculate mice. The original stock of virus (Sobrino et al., 1983) was amplified by passing it twice in ST cells and once in the BHK cell line. Supernatant was finally titrated ($TCID_{50}$) in BHK-21 monolayers before inoculation.

2.2. Cloning and expression of 2C and fragments

The 2C gene and the two fragments, 2C1 and 2C2, were all PCR amplified from an infectious clone of C-S8c1 (Nunez et al., 2001; Toja et al., 1999) using the following specific primers: For 2C: 5′-GAATTCGAATTCGCCGCCGCC**ATG**CTCAAAGCACGTGACATC-3′ (sense) and 5′-TCTAGATCTAGA**CTA**TTGCTTAAAAATTGGGTGGC-3′ (antisense). For 2C1: 5′-GAATTCGAATTCGCCGCCGCC**ATG**CTCAAAGCACGTGACATC-3′ (sense) and 5′-TCTAGATCTAGA**CTA**GTAACGTCGAAGTGGTC-3′ (antisense). For 2C2: 5′-GAATTCGAATTCGCCGCCGCC**ATG**AACCAGCAGACCGTTG-3′ (sense) and 5′-TCTAGATCTAGA**CTA**TTGCTTAAAAATTGGGTGGC-3′ (antisense). The fragments 2C1 and 2C2 encompassed nt 1-453 (aa 1-151) and 454-954 (aa 152-318) of the 2C gene, respectively. A start ATG codon (shown in bold) was introduced in the sense primers preceded by a Kozak motif (underlined) for improved translation and two *Eco*RI restriction sites for cloning. A stop codon (shown in bold) was introduced in the antisense primers, which also included two *Xba*I sites for directional cloning. Size of amplicons was verified by agarose gel electrophoresis in 1× TAE buffer. The 2C gene and fragments were ligated into pVAX1 (Invitrogen). Transformation and growth of bacteria

was performed as previously described by Barfoed et al. (2004a).

Presence and size of inserts was verified by restriction enzyme analysis. Sequence of inserts was determined by automatic sequencing of both strands using the ABI Prism BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and the ABI Prism 310 genetic analyser (Applied Biosystems). Protein expression was verified by transient transfection of PK15 cells with recombinant plasmids using FuGene 6.0 (Roche). Cells transfected with pVAX1 were used as negative control. After transfection, cells were incubated at 37 °C, 5% CO_2, for 24 h before fixation and staining. Transfected cells were stained using a FMDV specific guinea pig immune serum X-17 (Sorensen et al., 1998), prepared by repeated infections with different FMDV serotypes, and kindly provided by Dr. K.J. Sørensen, followed by horseradish peroxidase conjugated rabbit-anti-guinea pig Ig (DAKO, Denmark) and ethylcarbazole/H_2O_2. The recombinant plasmids containing 2C, 2C1 and 2C2 were designated pVAX1/2C, pVAX1/2C1 and pVAX1/2C2, respectively.

2.3. Prediction of CTL epitopes from C-S8c1 in Balb/c mice

Balb/c mice express the MHC I haplotype H2-Kd/H2-D^d The sequences of the C-S8c1 FMDV polyprotein (Toja et al., 1999) as well as the 2C gene alone were screened for potential H2-K^d 8-10-mer epitopes using the algorithms from the SYFPEITHI website (Institute for Cell Biology, Department of Immunology, Heidelberg, Germany, www.syfpeithi.de) (Rammensee et al., 1999) and the HLA peptide binding prediction website (BIMAS; Bioinformatics and Molecular Analysis Section, Center for Information Technology, National Institute of Health, USA, http://thr.cit.nih.gov/molbio/hla_bind/index.html) (Parker et al., 1994).

2.4. Preparation of gene gun cartridges

Cartridges for gene gun vaccination were prepared essentially as described by Barfoed et al. (2004b). In brief, plasmid DNA was coated onto gold microparticles (Bio-Rad, CA) with a diameter of 1.6 μm in the ratio of 2 μg DNA to 1 mg gold particles. Cartridges were prepared by coating Tefzel tubing (Bio-Rad) with the plasmid DNA coated gold particles and each cartridge contained approximately 1.4 μg DNA coated onto 0.5 mg gold particles.

2.5. Animal experiments

All animal experiments were performed according to national and institutional guidelines. Female Balb/c mice were purchased from Taconic Europe, Denmark. Mice were kept in isolation facilities at DFVF, Lindholm, and fed commercial diets and water ad libitum.

2.6. DNA vaccination and virus infection of mice

Plasmid DNA was purified using the EndoFree Mega plasmid preparation kit (Qiagen). Female Balb/c mice (8-weeks old at the beginning of the experiment) were vaccinated on the abdominal skin using the Helios gene gun system (Bio-Rad) giving two shots of 200 psi per vaccination. Prior to vaccination mice were anesthetized and shaved on the abdominal skin as described previously (Kamstrup et al., 2004). Four groups of mice were vaccinated three ($n = 20$ per group) or 4 times ($n = 5$ per group) with a 3-weeks interval with pVAX1 (control), pVAX1/2C, pVAX1/2C1 or pVAX1/2C2. To estimate the induction of specific CTL responses upon FMDV infection, 10 mice per vaccination group were inoculated by i.p. injection with 10^3 $CCID_{50}$ of the homologous FMDV isolate C-S8c1 on day 26 following third vaccination. At different days post-vaccination or post-infection (p.i.), animals (one mouse per group and time-point) were bled and their splenocytes used to determine activation of CTL. Mice to be tested were randomly selected from their respective vaccination groups. In addition, a control group of naïve, non-vaccinated mice were inoculated with virus as above and splenocytes from three animals per time-point were used for CTL determination.

2.7. Detection of infection

Blood samples were taken by tail vein puncture on days 1, 2 and 3 p.i., and the virus load present in the serum was estimated by titration on BHK-21 cells. All mice showing viremia on one or more days were scored as infected. Since the C-S8 c1 isolate has been reported to be pathogenic in Balb/c mice (Salguero et al., 2005), all mice were inspected twice a day for emergence of clinical signs. Moribund mice were euthanized. The presence of antibodies to FMDV capsid proteins in sera from inoculated mice obtained on day 14 p.i. was determined by ELISA based on the method described by Have et al. (1984).

2.8. In vitro stimulation of splenocytes

Splenocytes were isolated by chopping the spleen into small lumps and passage through a 70 μm nylon cell strainer (BD Falcon). Erythrocytes were lysed by incubation with ice-cold 0.83% NH_4Cl for 1 min. Splenocytes were washed twice with complete medium (RPMI 1640 (Gibco BRL), 10 mM HEPES (Gibco BRL), 50 μM β-2 mercaptoethanol (Sigma), streptomycin (100 μg/ml) and neomycin (50 μg/ml)) with 5% FCS and resuspended in complete medium with 10% FCS. Splenocytes were added to U-bottomed 96-well plates (NUNC, Denmark) (10^6 cells/well in 100 μl), mixed with stimuli (100 μl) and incubated at 37 °C, 5% CO_2 for 18 h. The following stimuli were used: 13 pools of each five pentadecamer peptides, covering the 2C protein with a 10 amino acids overlap (i.e. pool 1 contains peptides given by amino acid number: 1–15, 6–20, 11–25, 16–30 and 21–35; pool 2 contains peptides 26–40, 31–45, etc.), a peptide "KYKDAKEWL" (aa 62–70 of 2C), representing the nonamer CTL epitope with the highest binding score prediction for H2-K^d of the whole C-S8 c1 polyprotein, according to the SYFPEITHI algorithm and the fourth highest according to the BIMAS algorithm (Table 1), and the corresponding peptide from a type O1 (Manisa, Genbank accession no. AY593823 (Carrillo et al., 2005)) "KYKEAKEWL".

Table 1
Sequences predicted as CTL epitopes from both the 2C protein and the whole FMDV C-S8c1 isolate polyprotein by means of algorithms SYFPEITHI (Rammensee et al., 1999; access via www.syfpeithi.de) and BIMAS (Parker et al., 1994; access via http://thr.cit.nih.gov/molbio/hla_bind/index.html)

2C protein		C-S8cl polyprotein	
SYFPEITHI	BIMAS	SYFPEITHI	BIMAS
K_{62}KYKDAKEWL (2C1) BS = 28	K_{62}YKDAKEWL (2C1) BS = 3456000	K_{1164}YKDAKEWL(2C) BS = 28	E_{1512}YIEKANI (3A) BS = 5760000
V_{293}YQLVQEVI (2C2) BS = 24	G_{238}YKINNKLDI (2C2) BS = 2880000	V_{533}YNPPRTAL (VP3) BS = 27	A_{1684}YLVPRHL (3C) BS = 4800000
G_{182}FIPPMASL (2C2) BS = 22	V_{293}YQLVQEVI (2C2) BS = 2400000	Q_{230}YQNSMDTQL (VP4) BS = 26	Q_{230}YQNSMDTQL (VP4) BS = 41472000
K_{39}FVTMTDLV (2C1) BS = 21	G_{238}YKINNKL (2C2) BS = 2400000	E_{2011}YKFACQTFL (3D) BS = 25	K_{1164}YKDAKEWL (2C) BS = 3456000
G_{238}YKINNKLDI (2C2) BS = 21	K_{39}FVTMTDL(2C1) BS = 2304000	A_{829}YHKGPVTRL (VP1) BS = 24	Q_{603}YTGTINL(VP3) BS = 3456000

The five epitopes with highest binding score (BS) as predicted by each algorithm are shown, starting with the peptide giving the highest score at the top for each protein. Sequences of 8–10 mers and 9–10 mers are given from BIMAS and SYFPEITHI predictions, respectively. The N-terminal amino acid position (according to Toja et al., 1999) is indicated for epitopes predicted from the whole polyprotein, while this number refers to the position in 2C sequence for epitopes predicted from this single protein. The FMDV protein in which predicted epitopes are located (2C1 or 2C2 fragments for epitopes predicted in 2C) is indicated in parenthesis.

The C-S8 c1 peptide KYKDAKEWL was used to stimulate splenocytes from mice vaccinated with DNA or after FMDV challenge, while the O1 specific peptide KYKEAKEWL was exclusively used for stimulation after FMDV infection. Finally, the peptide "VYQLVQEVI", representing the nonamer CTL epitope with the second highest binding score for H2-K^d within 2C (aa 293–301 of 2C) was included in some of the assays. KYKDAKEWL resides in 2C1 while VYQLVQEVI resides in 2C2. Peptides and peptide pools were added to cells at a final concentration of 1 μg/ml for each peptide. All pentadecamers were synthesized by solid phase Fmoc chemistry, as previously described (Blanco et al., 2001; Garcia-Briones et al., 2004), while the nonamers were synthesised by Schafer-N (Copenhagen, Denmark). Stimulation with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml, Sigma–Aldrich, P8139) and ionomycin (500 ng/ml, Sigma–Aldrich, I0634) served as positive control. Cells incubated in medium only served as negative controls. After incubation for 18 h, monensin (M5273, Sigma–Aldrich), was added to all wells to a final concentration of 12 μM, and cells were incubated for 4 h, 37 °C, 5% CO_2, before staining. Plates with cells were spun for 3 min at 275 g, 4 °C, and medium removed.

2.9. Surface antigen and intracellular cytokine staining

Monoclonal antibodies for staining of cell surface antigens: PerCP-conjugated rat anti-mouse CD8a (BD#553036) and FITC-conjugated rat anti-mouse CD44 (BD#553133), were both diluted 1:200 in PBS with 5% FCS. Cells were incubated in the CD8/CD44 antibody solution for 30 min on ice and subsequently washed 3 times in PBS with 5% FCS and centrifugation for 3 min, 275 g at 4 °C. Thereafter, cells were fixed and permeabilized by resuspension in ice-cold 2% formaldehyde in PBS and incubation on ice for 20 min, and washed 3 times with PermWash (PBS with 1% FCS, 0.5% Saponin (S7900, Sigma–Aldrich) and 0.02% NaN_3). Washing was performed as above except that cells were incubated for 5 min in PermWash before each centrifugation. For detection of intracellular IFN-γ cells were stained with PE-conjugated rat anti-mouse IFN-γ antibody (BD#554412) diluted 1:100 in PermWash. All antibodies were obtained from BD Biosciences-Pharmingen (San Jose). Cells were incubated in IFN-γ antibody solution for 30 min on ice. Afterwards, cells were washed 3 times with PermWash as above, but with 5 min incubation before last centrifugation only. Finally, cells were resuspended in 0.74% formaldehyde in PBS, and kept in the dark at 4 °C until analysis.

2.10. Flow cytometry—acquisition and analysis

Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences-Pharmingen, San Jose). Five thousand $CD8^+$ cells were acquired per sample. Data analysis was performed using CellQuest Pro Version 5.1.1. Two criteria were set for scoring reaction to a given peptide or pool of peptides: (i) display of high-level CD44 (activation marker) and (ii) expression of IFN-γ. Thus, $CD8^+/CD44^{hi}$ T-cells that specifically expressed IFN-γ after ex vivo stimulation were considered peptide-specific CTLs.

In all Tables, results are presented as stimulation index (SI), calculated for each mouse and each individual stimulus from each mouse as the ratio between $CD8^+/CD44^{hi}$/IFN-γ^+ mononuclear cells for stimulated cells (for a given stimulus), over non-stimulated cells (medium only).

2.11. Statistical analysis

For statistical analysis of stimulation experiments, grouping of data was necessary. Experimental data as well as computer predictions indicated that the sequence KYKDAKEWL constituted the CTL epitope. For this reason, stimuli containing the sequence KYKDAKEWL or the corresponding KYKEAKEWL were grouped together, and compared with stimuli without these sequences, using standard ANOVA analysis (Mann–Whitney test).

3. Results

3.1. Prediction of potential CTL epitopes from FMDV

The whole FMDV polyprotein as well as the 2C gene alone were screened for potential H2-K^d epitopes using two different algorithms as described in Section 2. Several potential epitopes were identified and, in general, the two algorithms were in good agreement. According to both algorithms, the candidate CTL

epitope from 2C with highest H2-K^d binding score was a nonamer peptide: KYKDAKEWL. Furthermore, this epitope was predicted to be the best and the fourth best candidate H2-K^d epitope of the whole FMDV C-S8c1 polyprotein by the SYFPEITHI and BIMAS algorithm, respectively. Epitopes (octamer, nonamer or decamer) using the two algorithms predicted to have the five highest ranked binding scores are shown in Table 1.

These observations led us to address the presence of CTL epitopes in 2C for Balb/c mice by conducting DNA vaccination experiments with plasmid pVAX1/2C, expressing the entire 2C protein or plasmids pVAX1/2C1 and pVAX1/2C2, expressing the N-terminal or the C-terminal 50% of the 2C protein of the 2C protein, respectively.

3.2. Cloning and expression of 2C, 2C1 and 2C2

Three DNA plasmids containing the full-length 2C gene (pVAX1/2C), the first (pVAX1/2C1) and the second half (pVAX/2C2) of the gene, were generated by PCR amplification of the corresponding fragments using specific primers. These fragments were directionally cloned into the EcoRI and *Xba*I sites in pVAX1 and the incorporation of correct inserts was verified by digestion with the corresponding restriction enzymes, and by sequencing (see Section 2 for details). For each one of these plasmids, protein expression was demonstrated by in vitro transfection of PK15 cell monolayers and subsequent detection of FMDV-proteins using an FMDV specific guinea pig immune serum (Fig. 1). Finally, the plasmids were inoculated in Balb/c mice, to assess the immune responses elicited by 2C and its fragments.

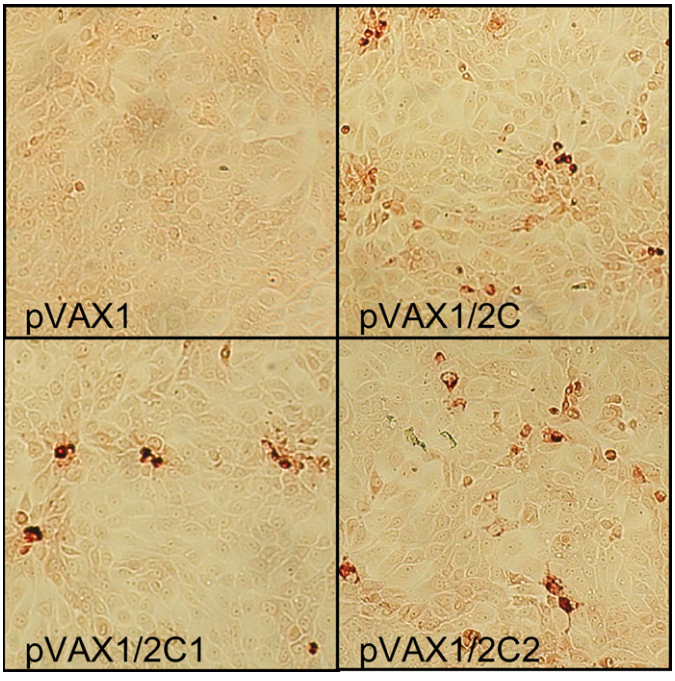

Fig. 1. Expression of FMDV 2C protein and fragments 2C1 and 2C2. The 2C gene and fragments thereof from FMDV type C isolate C-S8c1 were amplified by PCR and cloned into DNA vaccination vector pVAX1. PK-15A cells were transiently transfected with each individual plasmid, as indicated, and FMDV protein expression verified by staining of transfected, fixed cells with a polyclonal guinea-pig anti-FMDV antiserum.

Table 2
Amino acid sequence of the five 2C pentadecamer peptides (#11–15) included in pool 3

Pool 3
11: LEKQRDLNDPSKYKD
12: DLNDPSKYKDAKEWL
13: SKYKDAKEWLDNTRO
14: AKEWLDNTRQVCLKS
15: DNTRQVCLKSGNVHI

The predicted H2-K^d epitope KYKDAKEWL, fully included in peptides nos. 12 and 13, is underlined.

3.3. Identification of a CTL epitope in the FMDV 2C protein following DNA immunization

A preliminary experiment was performed in which mice were inoculated 3 times with pVAX1/2C. Splenocytes from vaccinated ($n = 1$), or naïve mice ($n = 1$), respectively, were stimulated in vitro with 13 pools of pentadecamer peptides covering the entire 2C protein, as detailed in Section 2. A specific increase in the number of IFN-γ producing cells from the vaccinated mouse was observed following stimulation with pool 3 (data not shown). No increase was observed following stimulation of splenocytes from the naïve mouse. Pool 3 included peptides nos. 11–15 corresponding to amino acids 51–85 of 2C. Interestingly, the predicted epitope KYKDAKEWL (covering aminoacids 62–70), was contained in both peptide 12 and peptide 13 from pool 3 (Table 2).

Having observed that the theoretical prediction and the ex vivo results coincided, a peptide with the sequence of the KYKDAKEWL epitope was synthesized and used for stimulation of splenocytes. Also, single peptides included in pool 3 were used for stimulation in the following experiments.

In a new experiment, groups of mice were DNA vaccinated with pVAX1/2C, pVAX1/2C1 or pVAX/2C2 as detailed in Section 2. The empty plasmid pVAX1, not expressing FMDV proteins, was administered to a group of control animals. Splenocytes from inoculated animals were incubated with the 13 pools of peptides, encompassing the entire 2C protein, with the single peptides from pool 3 (individual peptides no. 11–15), and with peptide KYKDAKEWL. Splenocytes were prepared for determination of post-vaccination CTL response on days 5 and 12 post third vaccination and on day 7 post fourth vaccination. One mouse was tested per vaccination group and time point.

Specific CTL responses were estimated as the increase in the number of CD8$^+$, CD44^{hi}, IFN-γ$^+$ cells after stimulation, relative to cells incubated in medium only, as described in Section 2. The response against pool 3, peptide 12 or KYKDAKEWL, and the medium background, on day 5 post third vaccination with either pVAX1 or pVAX1/2C is shown in Fig. 2. At all time points tested, splenocytes from 2C and 2C1 vaccinated mice responded strongly towards KYKDAKEWL, peptide 12, peptide 13 and pool 3 (Table 3 and data not shown). In general,

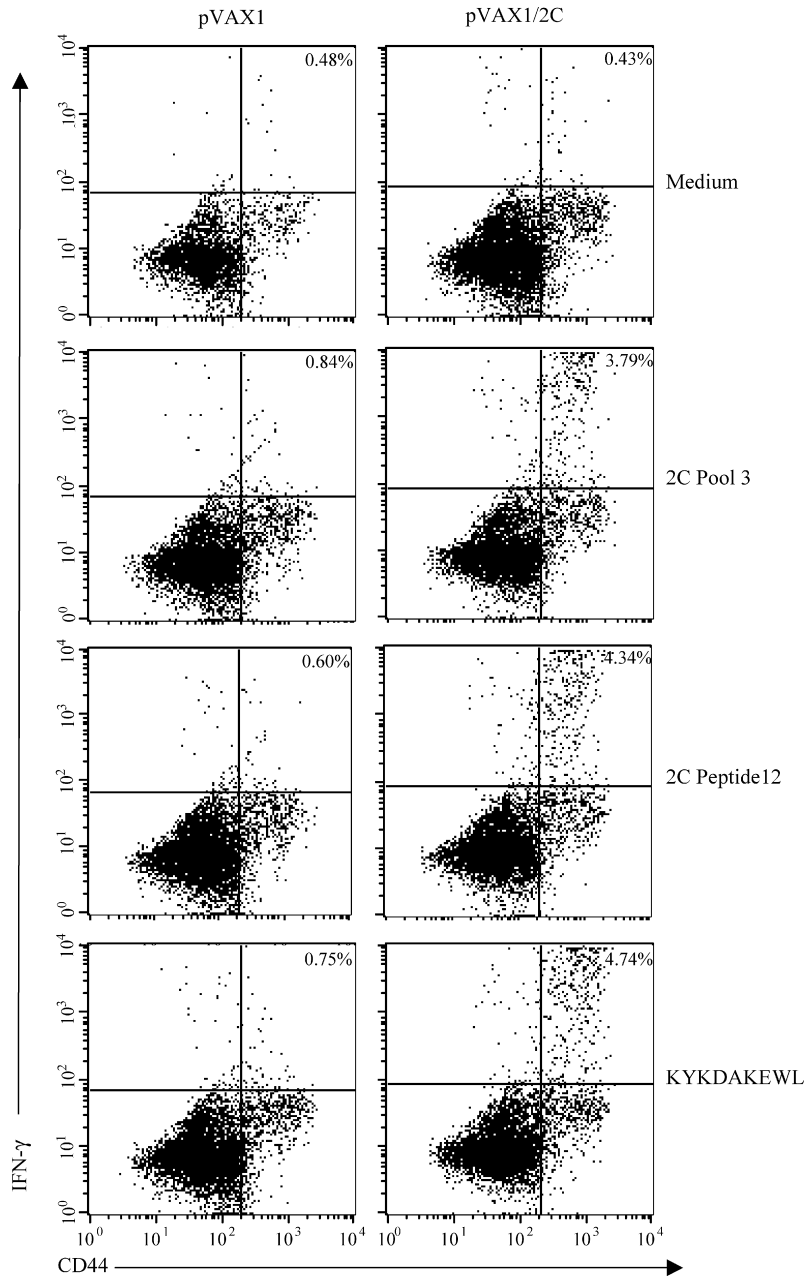

Fig. 2. Expression of IFN-γ and CD44 in CD8+ lymphocytes from vaccinated mice. Splenocytes from animals vaccinated with pVAX1 (control) or pVAX1/2C were collected on day 12 post third vaccination and stimulated in vitro with: medium (negative control), pool 3 peptides, peptide 12 alone or peptide KYKDAKEWL. Results correspond to cells gated as $CD8^+$. The percentage of $CD8^+$, $CD44^{hi}$, IFN-γ^+ cells of the gated mononuclear cells, is indicated in the upper right quadrant.

stimulation with peptide 12 resulted in stronger responses than those induced by peptide 13. Stimulation indices (SI) up to 10.42 were observed (Table 3).

When all KYKDAKEWL containing peptides were statistically treated as a group and their responses compared to those of other peptides not including this sequence motif, the difference was highly significant ($p = 0.0005$) for all mice vaccinated with 2C or 2C1. Conversely, and confirming the specificity of these responses, no difference between stimuli was observed with mock-vaccinated (PVAX1) or 2C2-vaccinated mice. Since KYKDAKEWL is contained in the 2C1 fragment of 2C, this is not surprising. The response increased between 5 and 12 days post third vaccination ($p < 0.05$), but no statistically significant difference was observed following three or four vaccinations (data not shown). Thus, among the predicted epitopes, KYKDAKEWL was the only peptide that induced significant in vitro stimulations in PBMC from immunized mice. No response was detected in either 2C- or 2C2-vaccinated mice towards peptide VYQLVQEVI that had been predicted the second best H2-K^d nonamer epitope within the 2C protein (Table 1), and which was included in the 2C fragment 2. Also, the response to pools containing the remaining predicted potential epitopes in 2C was below the level of significance (Table 3).

Table 3
Increase in IFN-γ producing cells on 12 days post third vaccination

Plasmid[a] Stimulus[b]	pVAX1	pVAX1/2C	pVAX1/2C1	pVAX1/2C2
KYKDAKEWL	**1.65**	**10.42**	**5.57**	**1.00**
PEPTID 11	1.27	1.21	1.46	0.89
PEPTID 12	**1.32**	**9.54**	**6.31**	**1.18**
PEPTID 13	**1.63**	**7.54**	**3.80**	**0.91**
PEPTID 14	1.56	1.14	1.00	0.82
PEPTID 15	1.54	0.97	1.37	0.93
POOL 1	1.85	1.03	1.51	1.29
POOL 2	1.43	0.77	1.06	1.36
POOL 3	**1.85**	**8.33**	**6.23**	**1.60**
POOL 4	1.63	1.03	1.17	1.24
POOL 5	1.12	1.65	1.77	1.02
POOL 6	1.34	1.03	1.60	1.31
POOL 7	1.25	1.01	1.29	1.22
POOL 8	1.38	1.14	1.34	1.02
POOL 9	1.38	0.95	1.23	1.24
POOL 10	1.52	1.19	1.20	1.00
POOL 11	1.08	0.86	0.77	1.42
POOL 12	0.86	0.92	1.31	1.00
POOL 13	1.41	0.92	1.11	0.93
Medium background (%)	0.46	0.46	0.35	0.45

Results are presented as stimulation index (SI), medium background is given as percentage $CD8^+/CD44^{hi}/IFN\text{-}\gamma^+$ cells. Stimulations with pool 3, peptide 12 and KYKDAKEWL shown for the pVAX1 and the pVAX1/2C vaccinated mice are based on the data shown in Fig. 2.

[a] Plasmid used for mouse inoculation.

[b] Stimuli shown in bold contain the predicted epitope KYKDAKEWL. Splenocytes were incubated overnight with the indicated stimuli (1 μg/ml).

Table 4
Increase of IFN-γ producing cells on 9 days post-infection in previously vaccinated mice

Plasmid[a] Stimulus[b]	pVAX1	pVAX1/2C	pVAX1/2C1
KYKDAKEWL	**1.23**	**1.98**	**1.57**
KYKEAKEWL	**1.21**	**1.88**	**1.58**
PEPTID 11	0.63	0.86	0.93
PEPTID 12	**0.94**	**1.98**	**1.48**
PEPTID 13	**1.01**	**1.44**	**1.03**
PEPTID 14	0.87	0.81	1.06
PEPTID 15	1.23	1.05	1.55
POOL 1	0.87	1.03	1.07
POOL 2	0.86	1.23	1.02
POOL 3	**1.03**	**1.62**	**1.38**
POOL 4	0.83	0.84	0.91
POOL 5	0.82	1.06	0.65
POOL 6	0.69	0.97	0.92
POOL 7	1.03	1.34	1.02
POOL 8	0.88	1.12	1.07
POOL 9	0.70	1.49	1.29
POOL 10	0.77	1.40	1.25
POOL 11	0.81	1.16	1.18
POOL 12	0.73	1.07	1.27
POOL 13	0.77	1.23	0.84
Medium background (%)	2.13	1.46	1.82

No viremia was detected in the mouse previously vaccinated with pVAX1/2C2, for which reason these data are not included. Splenocytes were incubated overnight with the indicated stimuli (1 μg/ml). Results are presented as stimulation index (SI), medium background is given as percentage $CD8^+/CD44^{hi}/IFN\text{-}\gamma^+$ cells. Stimulations with pool 3, peptide 12 and KYKDAKEWL shown for the pVAX1 and pVAX1/2C mouse are based on the data shown in Fig. 2.

[a] Plasmid used for mouse inoculation.

[b] Stimuli shown in bold contain the predicted C-S8c1 epitope KYKDAKEWL or that corresponding to 01 Manisa sequence KYKEAKEWL.

3.4. CTL responses against the KYKDAKEWL peptide are detected following FMDV infection of vaccinated and naïve mice

Part of the mice from each vaccination group (pVAX1, $n=7$; pVAX1/2C, $n=9$; pVAX1/2C1, $n=10$; and pVAX1/2C2, $n=7$) vaccinated a total of 3 times were inoculated with 10^3 $TCID_{50}$ of the homologous FMDV isolate on day 26 after last vaccination. Infection status was verified by testing for viremia and seroconversion and results from in vitro stimulation of splenocytes are presented only for those animals for which virus was detected in serum (Table 4). Post-infection response against KYKDAKEWL peptide was tested on days 9 (Table 4 and Fig. 3) and 16 p.i. (data not shown). In general, a higher background of IFN-γ producing cells was observed after infection, which resulted in a less efficient distinction of the specific stimulation induced by the different peptides relative to that achieved following vaccination only. The higher background of IFN-γ producing cells is believed to be induced by the FMDV infection (Ostrowski et al., 2005). However, all infected mice showed a statistically significant ($p<0.05$) increase of IFN-γ producing cells when stimulated with KYKDAKEWL and the corresponding pentadecamers, in comparison with the other peptides (Table 4; Fig. 3, and data not shown). This response was seen irrespective of the immunogen used for vaccination. The reaction observed against KYKDAKEWL by a previously pVAX1-vaccinated mouse, indicated that KYKDAKEWL could be recognized as an immunogenic epitope in the context of a natural infection.

In order to further investigate the CTL response against KYKDAKEWL induced by infection alone a small-scale experiment was carried out. Six naïve mice were infected and tested for reaction to a subset of peptides on days 14 and 21 p.i., three mice per day. A clear response against KYKDAKEWL was observed in two out of three mice on day 14 p.i., showing SI of 3.27 and 4.5, respectively (Table 5). The SI of the third infected mouse (I-1) was relatively low compared to the other two mice. On day 21 p.i., all three infected mice showed a consistent but generally weaker response than observed on day 14 p.i. These observations, together with those from pVAX1-vaccinated mice, indicate that KYKDAKEWL is a naturally occurring CTL epitope in Balb/c mice.

3.5. CTL responses against the KYKDAKEWL peptide from the C-S8c1 FMDV strain can recognize heterologous viral sequences

The majority of FMDV sequences reported differ only in one amino acid position from that of C-S8c1 in the KYKDAKEWL epitope, where most sequences display a conservative substitu-

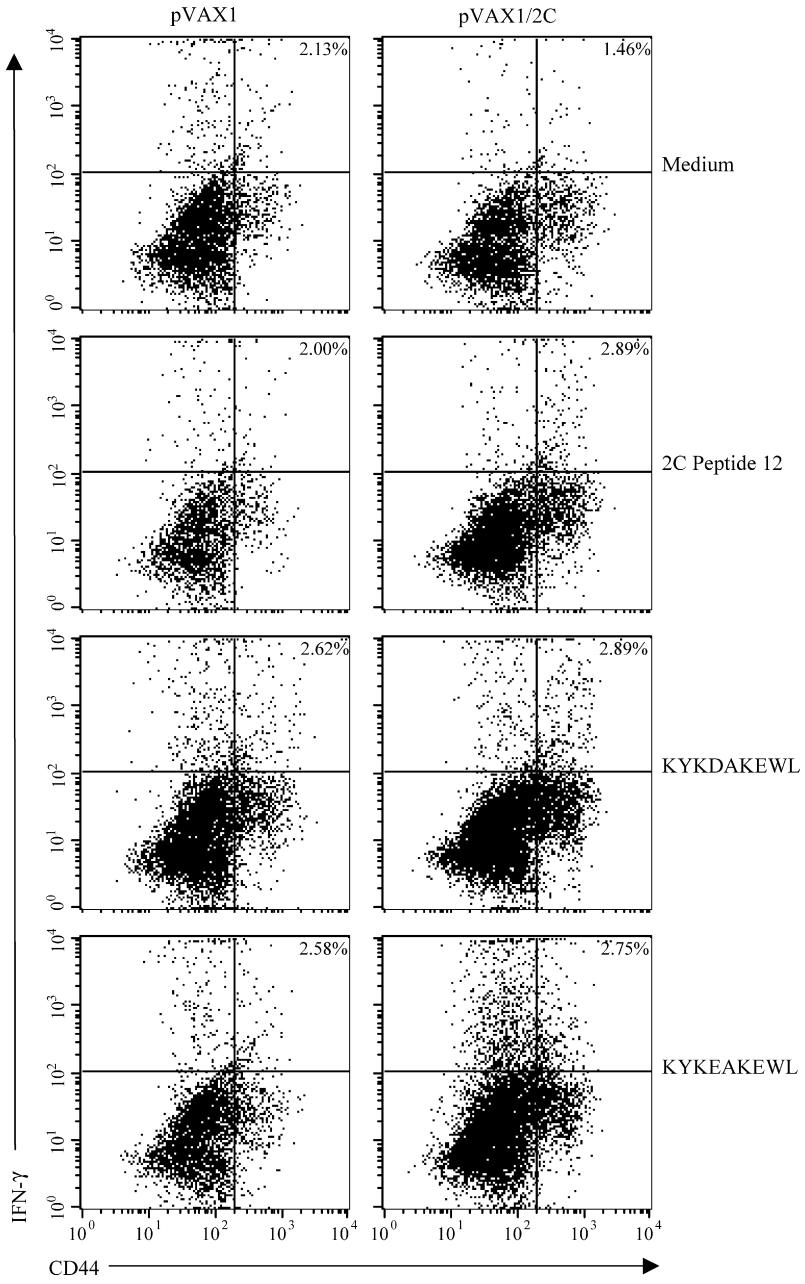

Fig. 3. Expression of IFN-γ and CD44 in CD8+ lymphocytes upon infection of vaccinated mice with FMDV isolate C-S8c1. Splenocytes, collected on day 9 p.i, were stimulated in vitro with: medium (negative control), peptide 12, peptide KYKDAKEWL or peptide KYKEAKEWL. Results correspond to cells gated as $CD8^+$. The percentage of $CD8^+$, $CD44^{hi}$, IFN-γ^+ cells of the gated mononuclear cells, is indicated in the upper right quadrant.

Table 5
Increase of IFN-γ producing splenocytes on days 14 and 21 post-infection (p.i.)

Mouse ID	Day 14 p.i.				Day 21 p.i.			
	C-1	I-1	I-2	I-3	C-2	I-4	I-5	I-6
KYKDAKEWL	1.49	1.68	3.27	4.50	1.00	1.46	1.39	1.50
Peptide 12	1.22	1.10	2.55	1.78	0.92	1.26	1.71	1.50
Peptide 13	0.90	1.16	2.55	1.50	0.93	1.12	0.94	1.21

Six mice (I-1 to I-6) were infected with 10^3 $TCID_{50}$ C-S8c1. Three mice were tested on each of the given time-points. Splenocytes were incubated overnight with the stimuli indicated (1 μg/ml). Splenocytes from one control mouse (non-infected) were included at each time-point (C-1 and C-2, respectively). Results are presented as stimulation index (SI).

tion at position four: an aspartic acid (E) instead of a glutamic acid (D). In order to test the potential cross-reactivity of the CTL response induced against KYKDAKEWL, splenocytes from mice previously vaccinated with pVAX1/2C, pVAX1/2C1 or pVAX1 and then infected with C-S8c1 FMDV, were stimulated with the corresponding heterologous peptide KYKEAKEWL. As shown in Table 4, this peptide was capable of inducing a response with SI equivalent to that induced by the homologous KYKDAKEWL. This result demonstrates that CTLs induced by sequences from a C-S8c1 FMDV strain (KYKDAKEWL) are able to recognize heterologous viral sequences such as KYKEAKEWL.

3.6. The induction of CTL responses against KYKDAKEWL peptide is insufficient to control FMDV infection in Balb/c mice

The protective capacity of the CTL responses induced by the vaccinations was tested by inoculation of vaccinated mice with FMDV. The viral dose used produced detectable infection in about 90% of inoculated animals which developed viremia and specific antibodies to FMDV structural proteins. The remaining animals were deemed uninfected (exclusion criteria: no viremia on any day tested, and no seroconversion) and were excluded from the analysis. Among those mice productively infected, the course of infection was evaluated by determination of virus load in serum at 48 h post-infection (optimal time point for the applied challenge dose, unpublished observations, S. Kamstrup), and by scoring for clinical disease and/or death. As shown in Table 6, virus load was consistent irrespective of the vaccination regimen, and the number of diseased or dead mice was not significantly affected by vaccination.

Table 6
Clinical observations in vaccinated mice upon FMDV challenge

Plasmid	Mouse	Viremia[a]	Clinical observation[b]
pVAX1	1	4.6	H
	2	6.3	D
	4	5.3	D
	6	6.7	D
	7	5.6	H
	9	6.1	D
	10	5.6	H
pVAX1/2C	1	5	H
	3	5	H
	4	6.1	S
	5	6.1	D
	6	6.2	D
	7	6.1	D
	8	5	D
	9	5	H
	10	6.1	D
pVAX1/2C1	1	1.7	H
	2	7.8[c]	D
	3	5.8	D
	4	6	D
	5	6.7	S
	6	6.1	D
	7	6.1	S
	8	6.7	D
	9	6.1	S
	10	6.7	D
pVAX1/2C2	2	7	D
	4	6.1	D
	5	5	D
	6	6.7[c]	D
	7	5.8	H
	9	3.3[c]	D
	10	4.4	H

Groups of mice were inoculated 3 times with the indicated plasmids. Twenty-six days after the last vaccination, mice were challenged by inoculation with 10^3 $TCID_{50}$ of homologous FMD virus.

[a] Viremia is given as log($CCID_{50}$/ml serum), as measured on day 2 p.i.

[b] H, healthy throughout observation period; S, diseased on one or more days; D, dead or moribund.

[c] No sample was available for 2 d.p.i., titer on 1 d.p.i. is given instead.

4. Discussion

The effector mechanisms leading to protective immunity to FMDV are not yet well understood. For a rational design of second-generation vaccines, elucidation of such mechanisms is required to induce optimal protective responses. While neutralizing antibodies have traditionally been considered to best correlate with FMDV protection, recent reports indicate that T-cell-mediated responses may also play a role in protection (Cedillo-Barron et al., 2001; Garcia-Briones et al., 2004; Sanz-Parra et al., 1999a,b).

A main problem in designing viral vaccines is the difficulty to get immune responses capable to confer protection against antigenically different strains. This is particularly true for RNA viruses with high mutational rates such as FMDV. One approach for the design of cross-protective FMDV subunit vaccines is the specific targeting of immune response towards epitopes with a high degree of inter- and intra-serotype conservation. Non-structural FMDV proteins are, in general, more conserved than structural proteins, and CTL responses are known to be effective against both structural and non-structural proteins. For these reasons, the induction of CTL responses to these proteins is an attractive possibility to achieve a broader immunity than that conferred by immunization against structural proteins.

Studies of cell-mediated responses against FMDV have so far been mostly aimed at identifying $CD4^+$ helper epitopes in outbred pigs and cattle (McCullough and Sobrino, 2004), and due to the outbred nature of the animals employed, identification of CTL epitopes has not been studied. By using inbred mice as a model we have identified an epitope that induces CTL activity after either viral infection or DNA vaccination. This epitope, KYKDAKEWL, was predicted by two different algorithms to efficiently bind the Balb/c MHC class I molecules of haplotype $H2\text{-}K^d$. Vaccination with truncated versions of the 2C protein induced CTL reactivity against this peptide, only when the immunogen contained KYKDAKEWL, confirming the specificity of the response. A time course study showed that reaction was clearly detectable at 5 days after third vaccination and had increased by 12 days after vaccination. No analysis was performed on earlier time points; so, whether two or fewer

immunizations would suffice for induction of CTL responses is currently unknown.

At each time point, one randomly selected mouse per vaccination group was sacrificed and splenocytes tested for their reactivity towards the full panel of FMDV 2C 15-mer peptides, KYKDAKEWL, and KYKEAKEWL. While this does not allow for statistical comparisons between vaccination groups at a given time point, we do observe the same pattern of reactivity on different time points within each vaccination group.

In addition, the four treatment groups include two receiving the KYKDAKEWL epitope (pVAX1/2C and pVAX1/2C1), and two not receiving this epitope (pVAX1/2C2 and pVAX1). For the individual mouse, grouping of stimuli according to KYKDAKEWL presence allowed meaningful statistical comparison of stimuli. In all experiments, reactivity to KYKDAKEWL in either context requires previous exposure to this epitope, confirming that this is indeed a CTL epitope.

Mice were tested for the presence of vaccination-induced antibodies in serum against 2C 20 days after vaccination, after vaccination and infection, or after infection alone. Since the in-house ELISA based on inactivated C-S8c1 virions does not detect antibodies specific for 2C, this was carried out by staining of PK15 cells transfected in vitro with the pVAX1/2C plasmid. While no antibodies could be demonstrated after infection alone, low levels of antibodies were detected after three vaccinations followed by infection or after four vaccinations with either of the plasmids pVAX1/2C, pVAX1/2C1 or pVAX1/2C2 (data not shown). Thus, while 2C is a good CTL inducer, and does indeed induce antibodies in natural hosts (Berger et al., 1990), it seems to be a poor B cell immunogen in Balb/c mice.

Although fairly well conserved among serotype C virus isolates, most FMDV isolates display a single amino acid difference in the KYKDAKEWL region (KYKEAKEWL instead of KYKDAKEWL). Some viruses show substitutions in other positions, but five out of the total of nine amino acids in this site were recently shown to be invariant in 103 FMDV isolates (Carrillo et al., 2005). Interestingly, the modified peptide stimulated CTL responses just as well as the parental peptide, against which the immune response was generated. Both aspartic acid (D) and glutamic acid (E) contain acidic side chains, differing only by one carbon in length. Furthermore, the modification is on position 4 in a nonamer epitope, leaving the for H2-K^d dominant anchor amino acids in position 2 and 9 unchanged (Coligan et al., 1996). Therefore, the change is minor and apparently not sufficient to influence either binding of the peptide to MHC class I or recognition of the MHC/peptide complex by the T-cell receptor. Although the cross reactivity has only been demonstrated one way, it seems reasonable to assume that it will also work vice versa. If so, an immune response to either epitope would have the same effect to viruses containing any of the two amino acid sequences.

FMDV infection in mice is very rapid. Under the challenge conditions used, peak viremia occurs 24–48 h after inoculation, and is usually cleared after 72 h (own unpublished observations). One contributing factor to the rapidity of viremia is the short infectious cycle time, i.e. the time from a cell is infected until progeny virus is released. This time has been estimated in vitro to be approximately 2–3 h (Quan et al., 2004), which defines the time frame within which a CTL response must take place to be fully protective. From this window must be subtracted time for the first viral peptides to be synthesized and presented by MHC class I molecules and probably also the late stages of virus infection where the cell may not even be sensitive to CTL-mediated killing, or where killing will still liberate infectious virus particles. Thus, a protective response needs to be immediate and highly effective in order to control an acute infection. On the other hand, potent FMDV specific CTLs may be efficient against persistent infection in carrier animals.

The response to the KYKDAKEWL epitope induced by DNA vaccination was at least as high as that induced by infection (Figs. 2 and 3, Tables 3 and 4). However, due to the elevated IFN-γ level in splenocytes from infected mice (irrespective of stimulus), direct comparison of response after vaccination or infection is not straightforward. The lower stimulation indices observed following infection, when compared to vaccination only, will be partly due to the higher background level, but immunosuppression as reported by Bautista et al. (2003) and Salguero et al. (2005) could also play a role. Vaccination with plasmids encoding the KYKDAKEWL epitope predisposed for a slightly higher response to peptides including this or the variant KYKEAKEWL epitope (Fig. 3; Table 4). Due to the limited number of animals tested, care should however be taken in interpretation of the post-infection data. Despite the high response detected after vaccination, no protection was achieved, since no statistically significant differences in virus load in blood, mortality, or disease frequency were noted. Different factors may contribute to the absence of protection. The time point of infection (26 days post third vaccination) may not have been optimal for the effector CTL response towards KYKDAKEWL. The response most probably already peaked and may be in the declining phase at the time point chosen. The kinetics of the post-vaccination effector response is currently being studied in order to infect mice at the peak time point. Furthermore, even though KYKDAKEWL specific $CD8^+$ T-cells recognize peptide coated target cells, they may be less efficient in recognizing virus-infected cells (work in progress). One reason for such an effect could be the massive down regulation of MHC class I molecules observed to be induced shortly following FMDV infection (Sanz-Parra et al., 1998).

In conclusion, we have here identified a murine CTL epitope that gives rise to a clear response following DNA vaccination or by infection with the serotype C FMDV isolate C-S8c1. The response detected was equally strong against the parental C-S8c1 epitope sequence, KYKDAKEWL, included in the vaccine, as towards the variant sequence, KYKEAKEWL, harboured by FMDV isolates from most of the other serotypes. The failure of the vaccination induced response to protect the mice against infection, does not exclude a role of CTL in FMDV, but emphasizes the need to further understand the interplay between CTL and B-cell responses in response to this virus infection. Inclusion of adequate combinations of B- and CTL-epitopes in synthetic vaccines, may help in enhancing the protection conferred by such vaccines.

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