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Identification of T-cell epitopes in the structural and non-structural proteins of classical swine fever virus

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To identify new T-cell epitopes of classical swine fever virus (CSFV), 573 overlapping, synthetic pentadecapeptides spanning 82% of the CSFV (strain Glentorf) genome sequence were synthesized and screened. In proliferation assays, 26 peptides distributed throughout the CSFV viral protein sequences were able to induce specific T-cell responses in PBMCs from a CSFV-Glentorf-infected d/d haplotype pig. Of these 26 peptides, 18 were also recognized by PBMCs from a CSFV-Alfort/187-infected d/d haplotype pig. In further experiments, it could be shown that peptide 290 (KHKVRNEVMVHWFDD), which corresponds to amino acid residues 1446–1460 of the CSFV non-structural protein NS2–3 could induce interferon-γ secretion after secondary *in vitro* restimulation. The major histocompatibility complex (MHC) restriction for stimulation of T-cells by this pentadecapeptide was identified as being mainly MHC class II and partially MHC class I. In cytolytic assays, CSFV-specific cytotoxic T-lymphocytes (CTLs) were able to lyse peptide 290-loaded target cells. These findings indicate the existence of a CSFV-specific helper T-cell epitope and a CTL epitope in this peptide.

Introduction

Classical swine fever virus (CSFV), a highly contagious pestivirus of the family *Flaviviridae* (Wengler, 1991), causes severe and lethal disease in domestic and wild pigs. The genome of CSFV consists of a positive-stranded RNA molecule of about 12·3 kb, which encodes a single open reading frame (ORF). The ORF is translated into a 3898 amino acid polyprotein (Meyers *et al.*, 1989), which gives rise to the different CSFV proteins after co- and post-translational processing. An autoprotease (N^{pro}) located at the N terminus of the polyprotein is followed by the structural proteins, core (C), E^{rns}, E1 and E2, and the non-structural proteins, p7, NS2–3, NS4A, NS4B, NS5A and NS5B (Meyers & Thiel, 1996).

Outbreaks of this disease occur frequently in several countries, usually with severe economic consequences (Edwards *et al.*, 2000; Stegeman *et al.*, 2000). Vaccination based on lapinized C strains of CSFV and temperature-sensitive mutants are being used in many parts of the world (Aynaud, 1988). However, these kinds of vaccines are prohibited from use within the European Union and several other countries due to the impossibility of differentiating between vaccinated and infected animals. In order to overcome the disadvantages of conventional vaccines, recombinant proteins (van Rijn *et al.*, 1996), virus vector vaccines (Rümenapf *et al.*, 1991; van Zijl *et al.*, 1991; Hooft van Iddekinge *et al.*, 1996), DNA vaccines (Andrew *et al.*, 2000) and peptide vaccines (DiMarchi *et al.*, 1986) have been proposed.

Synthetic peptides are promising candidate vaccines for the control of virus diseases. Peptide vaccines based on epitopes have been shown to induce a specific immune response (Deres *et al.*, 1989) and to protect the host against the disease (Bittle *et al.*, 1982; Menne *et al.*, 1997; Wiesmüller *et al.*, 1989). Thus, for the development of a synthetic peptide vaccine, characterization of the porcine immune response against CSFV is necessary. Studies on the cellular immune response to CSFV have been described previously (Kimman *et al.*, 1993). Viral proteins responsible for the induction of the virus-specific T-cell response were examined and a cytotoxic T-lymphocyte (CTL) epitope from the NS4A protein was identified (Pauly *et al.*, 1995). Also, the knowledge of allele-specific peptide motifs of major histocompatibility complex (MHC) class I (Falk *et al.*,

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1991) and class II (Falk *et al.*, 1994) molecules is of great importance for the prediction and identification of species-specific T-cell epitopes. The use of synthetic, overlapping peptides that span the regions encoding the viral proteins is one of the strategies for the identification of T-cell epitopes (Muller *et al.*, 1996; Blanco *et al.*, 2001; Ober *et al.*, 1998; Menne *et al.*, 1997). The aim of this study was to characterize the T-cell response to CSFV and to identify new CSFV T-cell epitopes using the cellular immune response of the natural host. A T-cell epitope mapping was performed with a set of 573 CSFV-derived, overlapping peptides spanning 82% of the single ORF of the genome. Further experiments showed that a peptide derived from the NS2–3 protein contained a helper T-cell epitope as well as a CTL epitope in its sequence.

Methods

■ **Infection of animals.** Two d/d haplotype NIH miniature swine (Sachs *et al.*, 1976) were infected by the intramuscular route (i.m.) with CSFV. Pig 1 was infected with 1×10^4 $TCID_{50}$ CSFV strain Alfort/187 and pig 2 was infected with 1×10^6 $TCID_{50}$ CSFV strain Glentorf. For the first and second challenge at monthly intervals, pig 1 was inoculated i.m. with 500 $TCID_{50}$ CSFV-Alfort/187 and pig 2 with 1×10^6 $TCID_{50}$ CSFV-Glentorf. Plasma and blood samples were collected before infection and eight times post-infection (p.i.).

■ **Monoclonal antibodies (mAb).** Murine mAbs against porcine MHC class I (mAb 74-11-10, mouse IgG_{2b}) (Pescovitz *et al.*, 1984), MHC class II (mAb MSA3, mouse IgG_{2a}) (Hammerberg & Schurig, 1986) and CD4 (mAb 74-12-4, mouse IgG_{2b}) (Pescovitz *et al.*, 1984) were kindly provided by J. K. Lunney (USDA, Agricultural Research Service, Beltsville, MD, USA). The porcine CD8 mAb (mAb 11/295/33, mouse IgG_{2a}) (Jonjic & Koszinowski, 1984) and mAb 'a18', a mouse IgG_{2a} directed against glycoprotein E2 of CSFV (Weiland *et al.*, 1990), were established at the Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany.

■ **Cells and viruses.** The Max cell line (Pauly *et al.*, 1995) used as target cells for the cytotoxicity assays was established at the Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany. The cell line was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 0·05 mM 2-mercaptoethanol, 10 mM HEPES (pH 7·2), 100 IU/ml penicillin and 0·1 mg/ml streptomycin sulphate. CSFV-Alfort/187 (Ruggli *et al.*, 1996) and CSFV-Glentorf (Pittler *et al.*, 1968) were a gift from R. Ahl, Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany. Both virus strains were propagated in the STE cell line (McClurkin & Norman, 1966) kindly provided by R. Ahl. Virus titres were determined by an indirect peroxidase-linked antibody assay using mAb a18 (Weiland *et al.*, 1990; Kosmidou *et al.*, 1995). The STE cell line was grown in 40% MEM–NEAA (Gibco BRL), 40% Leibovitz's L-15 medium (Gibco BRL), 10% tryptose phosphate broth, 10% horse serum, 100 IU/ml penicillin and 0·1 mg/ml streptomycin sulphate. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

■ **Synthesis and analysis of synthetic peptides.** A collection of 573 peptides spanning 82% of the amino acid sequence of the CSFV-Glentorf protein coding regions were synthesized using solid-phase multiple peptide synthesis (Jung & Beck-Sickinger, 1992) on a fully automated synthesizer (Syro). The 15-mer peptides, which overlapped each other by 10 residues, were prepared by solid-phase peptide synthesis using Fmoc/tBu chemistry. Syntheses were carried out on *p*-benzyloxybenzyl alcohol resin loaded with the first amino acid. The resin was distributed in 20–35 mg aliquots (15 μmol) to filter tubes positioned in a valve block. Fmoc deprotections were carried out with 50% piperidine in dimethylformamide (DMF). Washing steps were done with DMF. Double couplings (1 h each) were performed with Fmoc amino acids in 10-fold excess and 1-hydroxybenzotriazole/diisopropylcarbodiimide activation in DMF. Coupling reagents were filtered off and the resins were washed with DMF. Peptides were cleaved from the resins and side chain-deprotected with trifluoroacetic acid:phenol:ethanedithiol:thioanisole:water (82·5:5:2·5:5:5) for 3 h. Products were filtered from the resins and precipitated at −20 °C by the addition of diethylether. Precipitates were washed twice by sonication in diethylether and lyophilized from water:*tert*-butyl alcohol (1:4). The identity of each peptide was confirmed by electrospray mass spectrometry (triple-quadrupol, Micromass) and most of the peptides were greater than 70% pure, as determined by reverse-phase high-pressure liquid chromatography (Gynkotek) at 214 nm. All peptides (1 mg/ml) were dissolved in 1% DMSO in water as a stock solution (stored at −20 °C) and further diluted in culture medium.

■ **Isolation of PBMCs.** Infected pigs were bled every 1 or 2 weeks after the first infection. PBMCs were separated using density gradient centrifugation, as described previously (Summerfield *et al.*, 1996). Cells were frozen and stored in liquid nitrogen until use.

■ **Lymphoproliferation assays.** For the detection of CSFV-specific proliferation, PBMCs (10^5 cells per well) derived from CSFV-infected pigs were cultivated in MEM α-medium supplemented with 10% FCS, 2 mM L-glutamine, 0·05 mM 2-mercaptoethanol, 10 mM HEPES, 100 IU/ml penicillin and 0·1 mg/ml streptomycin sulphate as quadruplicates in 96-well round-bottom microplates (Greiner).

Cells were stimulated either with virus (10^4 $TCID_{50}$ CSFV-Alfort/187 or 5×10^4 $TCID_{50}$ CSFV-Glentorf per well) or with synthetic peptides (12·5 and 25 μg/ml). After incubation for 5 days at 37 °C in 5% CO_2, proliferative responses were determined by adding [*methyl*-^{3}H]thymidine for 18 h to the cultures (1 μCi per well) (Amersham). Cells were collected using a cell harvester system (Inotech). Incorporation of [^{3}H]thymidine was measured in a Microbeta 1450 β-counter (Wallac). Results were expressed as stimulation indexes (SI), defined as the mean c.p.m. of stimulated cultures/mean c.p.m. of control cultures. The response was considered to be positive when the SI was $\geqslant 2$.

■ **Inhibition of CSFV-derived peptide-specific proliferation.** For the determination of MHC restriction of the presentation of peptide 290, mAbs directed against the porcine CD4 (mAb 74-12-4), CD8 (mAb 11/295/33), MHC class I (mAb 74-11-10) and MHC class II (mAb MSA3) molecules were added to the cultures to test their capacity to inhibit peptide-specific proliferation of CSFV-primed PBMCs. Thus, PBMCs were incubated with the appropriate dilution of mAb (hybridoma supernatant anti-CD4, anti-CD8, anti-MHC class I or anti-MHC class II) for 2 h before use in proliferation assays, as described previously (Summerfield *et al.*, 1996).

■ **Cytotoxicity assays.** A chromium-release assay was used to measure CTL responses. CSFV-specific CTLs were generated by *in vitro* restimulation with virus (5×10^4 $TCID_{50}$ CSFV-Glentorf per well) for 6 days in 96-well round-bottom microplates.

MAX cells infected with CSFV-Glentorf at an m.o.i. of 1 for 48 h were used as target cells in cytotoxicity assays. Then, 1×10^6 target cells were labelled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham) for 1 h at 37 °C in 5% CO_2. After washing, cells were resuspended in cell culture medium. For CTL experiments with peptides, 4×10^4 non-infected chromium-labelled target cells were incubated at 37 ° C and 5% CO_2 with 20 μg of the

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respective peptides for 1 h. CSFV-infected or peptide-loaded target cells (1×10^3 cells per well) were added to various concentrations of effector cells (ranging from $1{\cdot}25 \times 10^4$ to 5×10^4 cells per well). All experiments were performed in triplicate cultures. Cells were centrifuged at 100 ***g*** for 5 min and incubated for 4 h at 37 °C in 5% CO_2. After pelleting the cells at 600 ***g*** for 10 min, the chromium levels in the supernatants were measured using a Cobra Autogamma γ-counter (Packard). The percentage of specific cytolytic activity was calculated as follows: [(c.p.m. experimental release) − (c.p.m. spontaneous release)/(c.p.m. total release) − (c.p.m. spontaneous release)] × 100. Mock-infected or negative peptide-loaded target cells served as the controls.

■ **Interferon (IFN)-γ ELISPOT assay.** The ELISPOT assay was used to enumerate CSFV- and peptide-specific IFN-γ-secreting cells. For this, 96-well nitrocellulose plates (Millipore) were coated with 100 µl per well of mouse anti-pig IFN-γ mAb (5 µg/ml) (Endogen) diluted in PBS. After overnight incubation at 4 °C, wells were washed with PBS and incubated with 200 µl of MEM α-medium for 1 h at room temperature. Different concentrations of PBMCs from CSFV-infected pigs (ranging from $1{\cdot}25 \times 10^5$ to 1×10^6 cells per well) were added in duplicate (each 100 µl) to the antibody-coated plates and incubated with 100 µl CSFV (2×10^4 $TCID_{50}$ CSFV-Alfort/187 or 5×10^4 $TCID_{50}$ CSFV-Glentorf per well) for 48 h. In peptide-sensitization experiments, 1×10^6 PBMCs per well (100 µl) were incubated with 100 µl of various concentrations of peptides (ranging from 5 to 100 µg/ml). PBMCs incubated with supernatant of mock-infected cells or irrelevant peptide 5 served as the negative controls.

After incubation at 37 °C in 5% CO_2 for 48 h, wells were washed with PBS and incubated with 100 µl of rabbit anti-pig IFN-γ polyclonal antibody (2·5 µg/ml) (Endogen) for 1 h at room temperature.

After washing with PBS, 100 µl per well of goat anti-rabbit peroxidase-conjugated IgG (Dianova) at a 1:200 dilution in PBS was added. After an additional 1 h of incubation at room temperature, wells were washed with PBS. For the development of IFN-γ-specific spots, 3,3′-diaminobenzidine (Sigma) was added for 4–7 min at room temperature. The colorimetric reaction was stopped by washing the plates with PBS and then water. After drying at room temperature, coloured spots were counted using a stereomicroscope at a 40-fold magnification. Analyses were repeated three times; the SE between these repeats was less than 10%.

Results

Proliferative responses of PBMCs to CSFV

Two inbred pigs (d/d haplotype) were infected with different strains of CSFV. To control the cellular immune response to the virus, blood samples were collected before and after (eight times at intervals of 2–3 weeks p.i.) infection. PBMCs were tested for their specific proliferative responses to CSFV in proliferation assays. PBMC proliferation was found to be both time- and dose-dependent (Fig. 1a and b, respectively). Specific responses of PBMCs from CSFV-infected pigs were observed at about 30 days p.i. No stimulation was observed with PBMCs collected from the swine prior to infection (day 0). An optimal stimulation of PBMCs could be induced with virus at an m.o.i. of 0·16–2·5 for strain Alfort/187 and at an m.o.i. of 0·1–4 for strain Glentorf. Results from kinetic experiments suggest that the culture conditions were optimum when [^{3}H]thymidine incorporation was initiated 5 days after stimulation with CSFV (Fig. 1c). PBMCs from the non-infected

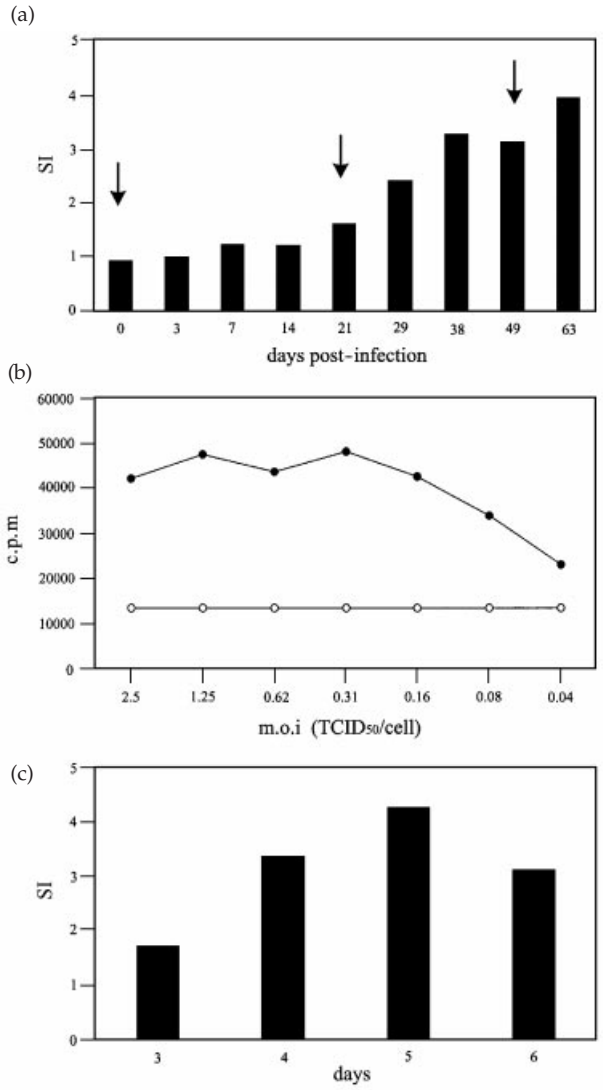

Fig. 1. Proliferative responses of PBMCs to CSFV. (a) Time-course study of the cellular immune response against CSFV. PBMCs derived from a CSFV-Alfort/187-infected pig collected at different days p.i. were restimulated *in vitro* with CSFV at an m.o.i. of 1. The proliferation of PBMCs before infection (day 0) served as the control. The day of infection is shown by an arrow. (b) Dose-dependent proliferative responses of CSFV-primed PBMCs. Proliferation values of PBMCs from a CSFV-Alfort/187-infected pig in the presence of different doses of virus (●). The experiment was carried out with an initial m.o.i. of 2·5. Spontaneous proliferation of PBMCs served as the control (○). (c) Time-dependent proliferative responses after stimulation *in vitro* with CSFV. PBMCs from a CSFV-Glentorf-infected pig were incubated with CSFV at an m.o.i. of 0·5. The proliferation of PBMCs was determined from day 3 to day 6 with an 18 h [^{3}H]thymidine-incorporation assay. PBMCs incubated with supernatant of mock-infected cells served as the negative control.

pigs showed no specific CSFV proliferation in the presence of CSFV.

Proliferative responses of PBMCs to CSFV-Glentorf-derived peptides

To characterize the T-cell epitopes responsible for CSFV antigenicity, a set of 573 pentadecapeptides, each one

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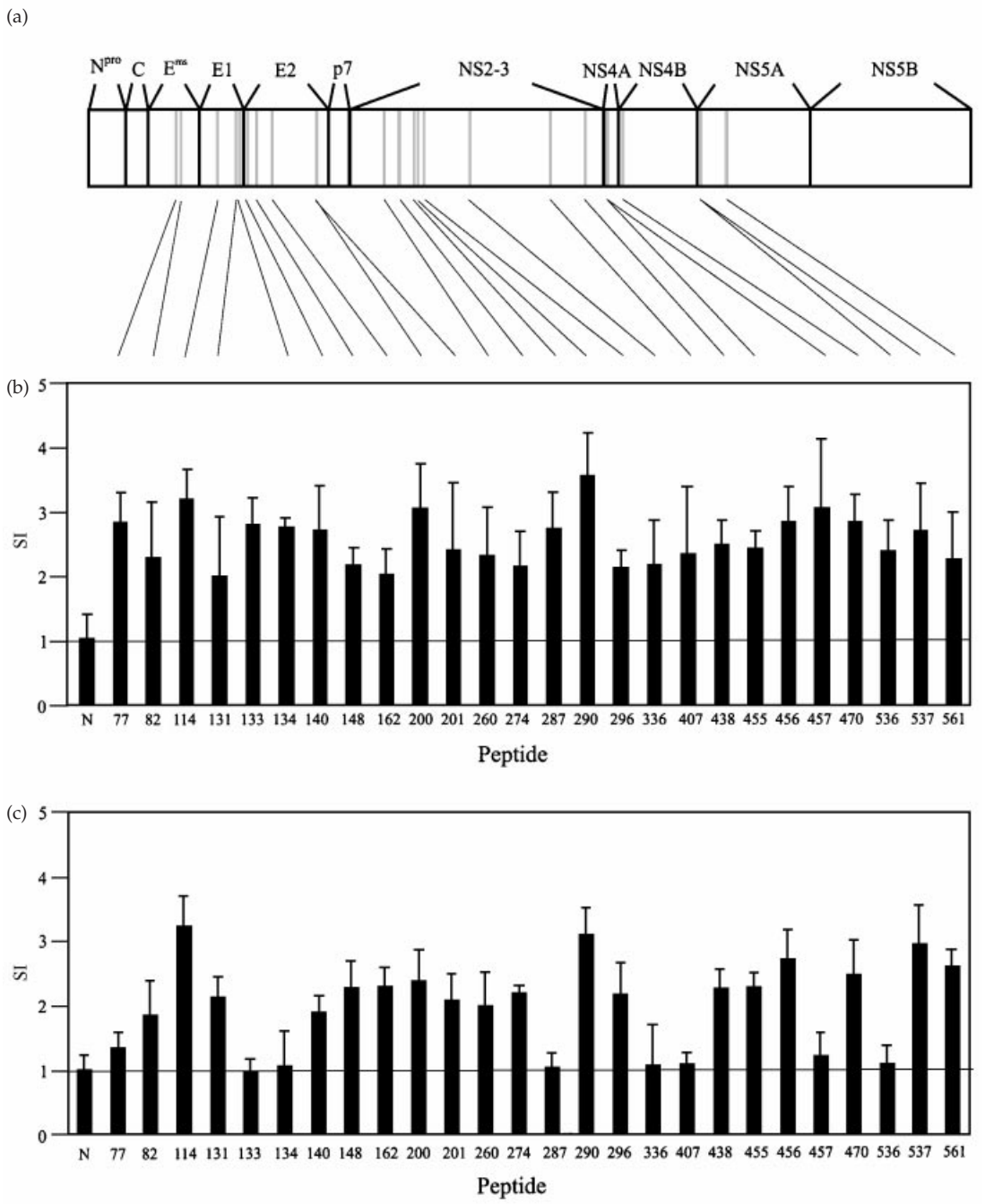

Fig. 2. Proliferative responses of PBMCs from CSFV-infected, d/d haplotype, inbred pigs to synthetic pentadecapeptides of CSFV. (a) A schematic representation is shown of the ORF of CSFV genomic RNA (Meyers & Thiel, 1996). The locations of the stimulating peptides in the viral polyprotein are shown. (b) PBMCs at 76 days p.i. from a CSFV-Glentorf-infected pig were tested for *in vitro* proliferation to 573 synthetic peptides (12·5 and 25 μg/ml). The SI of 26 peptides, which were identified as T-cell epitopes in proliferation assays, are plotted against the peptide number. (c) The stimulating peptides were tested further with PBMCs at 63 days p.i. from a CSFV-Alfort/187-infected pig. The irrelevant peptide 5 (N, EEPVYDATGRPLFGD) served as the negative control. Results represent the SI at the peptide concentration giving optimal stimulation. The error bars indicate SD. The sequences of the peptides are summarized in Table 1.

overlapping the previous one by 10 amino acids, was synthesized. The peptides covered the amino acid sequence of the following CSFV-Glentorf proteins: N^{pro}, C, E^{rns}, E1, E2, p7, NS2–3, NS4A, NS4B and part of NS5A. The *in vitro* proliferative response of PBMCs to the CSFV peptides was studied at 76 days p.i. for a pig infected with CSFV-Glentorf (Fig. 2b) and 63 days p.i. for a pig infected with CSFV-Alfort/187 (Fig. 2c). Results of [^{3}H]thymidine incorporation

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Table 1. Differences in the primary amino acid sequence of the stimulating peptides between CSFV strains Glentorf and Alfort/187

The 26 sequences containing T-cell epitopes for CSFV-Glentorf, as identified in proliferation assays, are shown. Stimulating peptides for both infected pigs are underlined, whereas differences in the amino acid sequence of strains Glentorf and Alfort/187 are indicated in bold. The amino acid positions spanned by each peptide in the CSFV polyprotein and the genetic location of the peptide in each viral CSFV protein are shown. The underlined amino acid sequences indicate the CTL epitope described by Pauly *et al*. (1995).

Peptide no.	Residues	Location	Amino acid sequence (strain Glentorf)	Alfort/187
77	381–395	E^{rns}	C R Y D K D A D **I** N V V T Q A	**I** → V
82	406–420	E^{rns}	K K G K N F S F A G T **I** I E G	**I** → V
114	566–580	E1	T A S A L Y L I **S Y** Y V I P Q	**SY** → LH
131	651–665	E1	T R V W N S A S T T A F L I C	
133	661–675	E1	A F L I C L I K V L R G Q I V	
134	666–680	E1	L I K V L R G Q I V Q G V I W	
140	696–710	E2	D Y R Y A I S S T N E I G L L	
148	736–750	E2	I C **A** A G S F K V T A L N V V	**A** → V
162	806–820	E2	T T L L N G S A F Y L V C P I	
200	996–1010	E2	Y E P R D S Y F Q Q Y M L K G	
201	1001–1015	E2	S Y F Q Q Y M L K G E Y Q Y W	
260	1296–1310	NS2–3	T L I L I L P T Y E L T K L Y	
274	1366–1380	NS2–3	K A I L I S C I S N K W Q F I	
287	1431–1445	NS2–3	K K F F L L S S R V K E L I I	
290	1446–1460	NS2–3	K H K V R N E V M V H W F **D** D	**D** → G
296	1476–1490	NS2–3	A T L S K N K **N** C I L C T V C	**N** → H
336	1676–1690	NS2–3	V V C Q S N N K M T D E S E Y	
407	2031–2045	NS2–3	S Q S P Y V V V V A T N A I E S	
438	2186–2200	NS2–3	E P I Q L A Y N S Y E T Q V P	
455	2271–2285	NS2–3–NS4A	G L S T A E N A L L V A L F G	
456	2276–2290	NS4A	E N A L L V A L F G Y V G Y Q	
457	2281–2295	NS4A	V A L F G Y V G Y Q A L S K R	
470	2346–2360	NS4B	E A M T N Y A **K** E G I Q F M K	**K** → R
536	2676–2690	NS4B–NS5A	S E G K I R Q L S S N Y I L E	
537	2681–2695	NS4B–NS5A	R Q L S S N Y I L E L L Y K F	
561	2801–2815	NS5A	V E L Y Y K G **T** T I K L D F N	**T** → A

assays showed that stimulation with CSFV-derived peptides induces a significant proliferation of PBMCs between day 4 and day 5 post-stimulation. The optimal time for determination of the proliferative response of PBMCs was day 5 (data not shown). The proliferative responses of PBMCs to the CSFV-derived peptides proved to be dose-dependent and, after titration experiments (data not shown), 12·5 and 25 μg/ml of each pentadecapeptide were tested in proliferation assays. A peptide with an SI $\geqslant 2$ was identified as stimulatory. For the CSFV-Glentorf-infected pig, 26 of 573 peptides induced a significant proliferative response in PBMCs compared to spontaneous proliferation (Fig. 2b). A total of 18 peptides stimulated PBMCs from both animals infected either with CSFV-Glentorf or with CSFV-Alfort/187 (Fig. 2 and Table 1). With PBMCs isolated prior to infection, no specific reactivity to the stimulating peptides was detected. A variation between the proliferative responses of PBMCs from the pigs to the individual peptides was observed (Fig. 2). The highest levels of specific proliferation of PBMCs to some peptides were found for the animal infected with CSFV-Glentorf. The location of the T-cell epitopes on the CSFV genome is shown in Fig. 2(a). CSFV-Glentorf (GenBank accession no. U45478) and CSFV-Alfort/187 (GenBank accession no. X87939) showed 98·2% identity with respect to the amino acid sequence of the peptides. Interestingly, peptides 77 (E^{rns}), 82 (E^{rns}), 114 (E1), 148 (E2), 290 (NS2–3), 296 (NS2–3), 470 (NS4B) and 561 (NS5A) showed differences in amino acid sequence between strains Glentorf and Alfort/187 (Table 1). In spite of the difference in their sequence, most of the peptides were able to induce significant proliferation of PBMCs from both animals. Only peptide 77 (E^{rns}) showed a lack of stimulating capacity with PBMCs from swine infected with CSFV-Alfort/187. A difference of one amino acid in the sequence between these two strains (Table 1) could be the reason for loss of the

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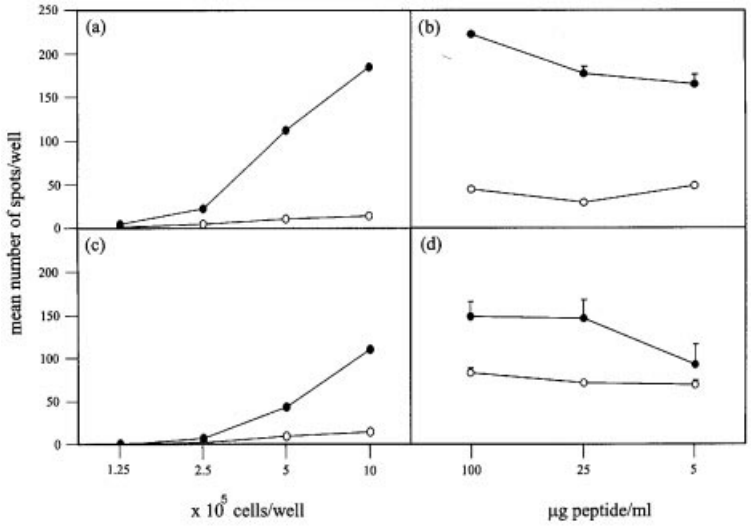

Fig. 3. IFN-γ secretion after CSFV (a, c) or peptide (b, d) stimulation using ELISPOT assay. IFN-γ-secreting cells obtained from PBMCs of (a) CSFV-Glentorf or (c) CSFV-Alfort/187-infected pigs incubated with the respective strain of the virus were quantified by ELISPOT assay. PBMCs were seeded at different concentrations (ranging from $1{\cdot}25 \times 10^5$ to 1×10^6 cells per well) in duplicate and were incubated with (a) CSFV-Glentorf (5×10^4 $TCID_{50}$ per well, ●) or (c) CSFV-Alfort/187 (2×10^4 $TCID_{50}$ per well, ●). PBMCs incubated with medium from mock-infected cells served as the negative control (○). The number of cells secreting IFN-γ in response to peptide 290 is shown (b, d). IFN-γ-secreting cells were obtained from (b) a CSFV-Glentorf or (d) a CSFV-Alfort/187-infected pig. PBMCs (10^7 cells/ml) were seeded in duplicate and incubated with different concentrations of peptide 290 (5, 25 and 100 µg/ml, ●). PBMCs incubated with an irrelevant peptide served as the control (○). The error bars in (d, b) indicate the SD.

stimulating capacity of this peptide. The behaviour of peptides 133 (E1), 134 (E1), 287 (NS2–3), 336 (NS2–3), 407 (NS2–3), 457 (NS4A), 536 (NS4B–NS5A) was conspicuous: these peptides were not able to stimulate PBMCs derived from the CSFV-Alfort/187-infected pig although they showed no difference in the amino acid sequence between strains Glentorf and Alfort/187.

Peptide 290, which is derived from NS2–3, plays an immunodominant role in both animals and always showed the highest stimulation capacity in proliferation assays.

Secretion of IFN-γ

To confirm the reactivity of CSFV-specific T-cells against CSFV antigens, porcine T-cells were tested for their ability to produce IFN-γ upon virus as well as peptide stimulation in ELISPOT assays. These assays enable the detection of IFN-γ release on a single cell level and a determination of T-cell frequencies. First, the number of cells releasing IFN-γ was detected by spot formation in the presence of different strains of CSFV (Fig. 3a, c). Various concentrations of PBMCs (ranging from $1{\cdot}25 \times 10^5$ to 1×10^6 cells per well) from CSFV-infected swine were tested. The mean number of spots increased with an increasing amount of cells. For spot detecting, 1×10^6 cells per well were considered to be appropriate. The release of IFN-γ was CSFV-specific, as PBMCs incubated with supernatant from mock-infected cells presented only a few spots. High numbers of IFN-γ-secreting cells were observed when PBMCs from infected pigs were stimulated with peptide 290 (Fig. 3b, d). The number of cells releasing IFN-γ in response to peptide 290 was higher with PBMCs from swine infected with CSFV-Glentorf compared with the PBMCs from swine infected with CSFV-Alfort/187. The release of IFN-γ by peptide 290 was shown to be sequence-specific, as only a few spots were observed after incubation of PBMCs with an irrelevant peptide. These results are an additional indication for an immunodominant role of peptide 290.

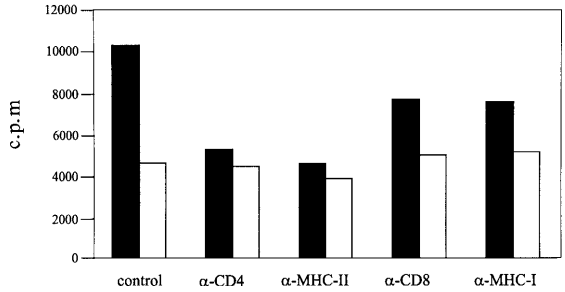

Fig. 4. MHC restriction of the CSFV peptide-specific T-cell proliferation. PBMCs (10^5 cells per well) from a CSFV-Glentorf-infected pig were stimulated with either peptide 290 (25 µg/ml, black) or an irrelevant peptide (25 µg/ml, white) as antigens in the presence of murine mAbs specific for CD4, CD8, MHC class I or MHC class II molecules. Cultures incubated with peptide 290 (black, control) or irrelevant peptide (white, control) without specific antibodies served as the controls. The proliferation of the cells was quantified in triplicate by [^{3}H]thymidine incorporation.

MHC restriction of the lymphoproliferative response to CSFV peptide 290

Virus-specific $CD8^+$ CTLs recognize viral peptides presented by MHC class I molecules. In contrast, the peptide MHC class II complexes are recognized by virus-specific $CD4^+$ helper T-cells. This interaction between T-cell receptor, MHC and accessory molecules (CD4 and CD8) can be blocked by the addition of mAbs against these surface molecules. To analyse the MHC restriction of the proliferative response to peptide

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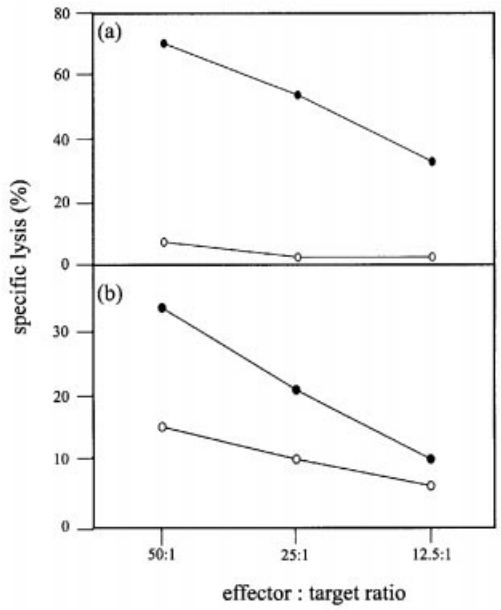

Fig. 5. Specific cytotoxicity of CSFV CTL to target cells sensitized by (a) CSFV or (b) CSFV-derived peptide 290. PBMCs of a CSFV-Glentorf-infected pig restimulated *in vitro* with virus (5×10^4 $TCID_{50}$) were used as effector cells. The cytotoxicity of the T-cells was determined in a chromium-release assay by lysis of (a) virus-sensitized (●, m.o.i. of 1) or (b) peptide-sensitized (●, 20 μg peptide/4×10^4 target cells) MAX cells as targets. MAX cells incubated with supernatant of mock-infected cells (a, ○) or with irrelevant peptide 5 (b, ○) were used as the controls.

290, we used mAbs against CD4, CD8, MHC class I and MHC class II molecules. The specific proliferative response could be blocked by an anti-MHC class II mAb as well as by an anti-CD4 mAb (Fig. 4). However, a lower level of inhibition of proliferation using anti-CD8 and MHC class I antibodies could also be observed. In conclusion, peptide 290 was able to stimulate helper T-cells as well as $CD8^+$ cytotoxic T-cells.

CSFV-specific CTL response

To analyse the cytolytic activity of CSFV-specific CTLs, virus-restimulated PBMCs derived from the CSFV-Glentorf-infected pig were tested for their ability to lyse target MAX cells (Pauly *et al.*, 1995). Their activity was determined by the lysis of chromium-labelled CSFV-infected or peptide 290-loaded target cells (Fig. 5). For the generation of CSFV-specific CTLs, PBMCs derived from the infected animals were cultivated for 6 days *in vitro* in the presence of CSFV. These CTLs elicited a strong cytolytic activity against target cells infected previously with the virus (Fig. 5a). Furthermore, a significant cytolytic response against peptide 290-loaded MAX cells was observed (Fig. 5b). Mock-infected target cells or target cells loaded with an irrelevant peptide (peptide 5), which were both used as negative controls, were not lysed by the CSFV-specific CTL effector cells. The peptides were non-toxic to the cells, as the level of spontaneous chromium release of target cells incubated with or without the irrelevant peptide was similar. In conclusion, target cells incubated with peptide 290 were recognized by CSFV-specific CTLs. Peptide 290 presented by d/d haplotype MHC class I molecules, which was characterized as MHC class II-restricted, could also be detected by CSFV-specific CTLs.

Discussion

The study of the cellular immune response to CSFV in the natural host is of utmost importance for understanding the interaction between the pathogen and the swine immune system. This is the basis for the development of vaccines to control virus infection. In this work, virus-specific T-cell responses were studied using PBMCs derived from CSFV-infected d/d haplotype swine, which were restimulated *in vitro* either by CSFV or by synthetic peptides. Pentadecapeptides were selected for use, as MHC class II molecules bind peptides of 10–30 amino acids in length (Chicz *et al.*, 1993). Screening of 82% of the amino acid sequence of CSFV using these overlapping pentadecapeptides yielded 26 stimulating peptides, when investigated with PBMCs from a CSFV-Glentorf-infected pig. Eleven stimulating peptides were located in the region encompassing the structural proteins E^{rns}, E1 and E2 and 15 peptides were identified on the non-structural proteins NS2–3, NS4A, NS4B and NS5A. The stimulating peptides 455 and 456 on the non-structural protein NS4A were reported previously to contain an MHC class I-restricted epitope (Pauly *et al.*, 1995). Obviously, E^{rns}, E1, E2, NS2–3, NS4A, NS4B and NS5A proteins are important targets for the cellular immune response, since peptides derived from these proteins showed a CSFV-specific immune response. No stimulating peptides could be identified on the proteins N^{pro}, C and p7.

Of the 26 stimulating peptides derived from CSFV-Glentorf proteins, 18 induced a proliferative response in lymphocytes from a CSFV-Alfort/187-infected pig.

Of interest is the finding that the MHC d/d haplotype recognized common T-cell epitopes in both inbred swine. Otherwise, some peptides (e.g. peptides 133 and 134) with an identical amino acid sequence between both strains (Glentorf and Alfort/187) were recognized as T-cell epitopes only with PBMCs from one pig. This MHC-independent preference for epitopes may have been caused either by host factors or by specific virus factors. This point might be the content of further studies.

Five of the common T-cell epitopes (peptides 140, 148,162, 200 and 201) were located on the E2 protein. The E2 glycoprotein is the most antigenic glycoprotein of CSFV and an immune response against E2 alone was reported to be sufficient for protection against CSFV (van Zijl *et al.*, 1991; Hulst *et al.*, 1993; König *et al.*, 1995). Our findings confirmed the striking antigenicity of this viral protein to induce an immune response. The stimulating peptides 200 and 201 derived from the E2 region are part of a B-cell epitope described previously (Yu *et al.*, 1996). The overlap of B- and T-cell epitopes in the same protein region has been described for

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other viral antigens (Rodriguez *et al.*, 1994). Peptide vaccines encompassing B- and T-cell epitopes can result in the enhancement of antibody responses (Borrás-Cuesta *et al.*, 1987; Collen *et al.*, 1991).

The pentadecapeptide 290, which is derived from NS2–3, showed the highest SI in all proliferation assays performed. The reactivity of PBMCs against this peptide was thus characterized further. To analyse the virus- and peptide-inducible production of IFN-γ from T-cells on a single cell level, ELISPOT assays were carried out. Advantages of the ELISPOT assay include sensitivity and efficiency in the detection of antigen-specific T-cells at the single cell level (Tanguay & Killion, 1994). However, T-cell responses detected with this assay are limited to the T-cells that secrete IFN-γ. This assay represents a useful tool for the *ex-vivo* determination of frequencies of antigen-specific T-cells. It is known that $CD4^+$ T-cells (Th1) as well as $CD8^+$ T-cells are able to produce IFN-γ. In this study, virus-specific (CSFV-Glentorf and -Alfort/187) and peptide-specific (peptide 290) IFN-γ release were detected by ELISPOT assay.

In order to characterize peptide 290-specific T-cells, the MHC restriction of the proliferative response to this peptide was determined. Proliferation in the presence of peptide 290 was inhibited principally by anti-MHC class II and anti-CD4, confirming an MHC class II restriction. However, a partial inhibition of proliferation of PBMCs was also observed with anti-MHC class I and anti-CD8 antibodies.

This MHC class I restriction of the pentadecapeptide 290 was tested in CTL assays for the capacity of the peptide to elicit cytotoxic T-cell responses. Virus-specific CTLs were tested against MAX cells loaded with peptide 290. The results indicated the presence of a CTL epitope in the sequence of peptide 290. Thus, peptide 290 has the capacity to prime both MHC class I and class II-restricted T-cells. The sequence encoded a helper T-cell and a CTL epitope, representing a multideterminant peptide. This protein region spanning amino acids 1446–1460 (peptide 290) is highly conserved among the different CSFV strains.

Lytic activities of CTLs against target cells infected with CSFV were higher than those found against target cells loaded with peptide 290. Indeed, this fact can be explained due to the presence of additional and more active CTL epitopes on the virus. CTLs usually recognize peptide fragments from 8 to 11 amino acid residues in length bound to an MHC class I molecule (Falk *et al.*, 1991), but it has also been described that longer peptides could be recognized by CTLs (Bertoletti *et al.*, 1993; Pauly *et al.*, 1995).

In conclusion, we have defined new T-cell epitopes on CSFV proteins. Peptide 290 on the NS2–3 protein has the capacity to elicit both $CD4^+$ and $CD8^+$ T-cell responses, suggesting that this protein segment may be a potential candidate for a synthetic peptide vaccine. Induction of CTL responses can be potentiated by covalent linkage of CTL and helper T-cell epitopes (Partidos *et al.*, 1996; Shirai *et al.*, 1994; Stuhler & Walden, 1993). Candidate sequences for synthetic peptide vaccines should be conserved among different CSFV strains, binding diverse MHC molecules (Kubo *et al.*, 1994; Oldstone *et al.*, 1992) and contain helper T-cell, CTL and B-cell epitopes (An & Whitton, 1997).

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