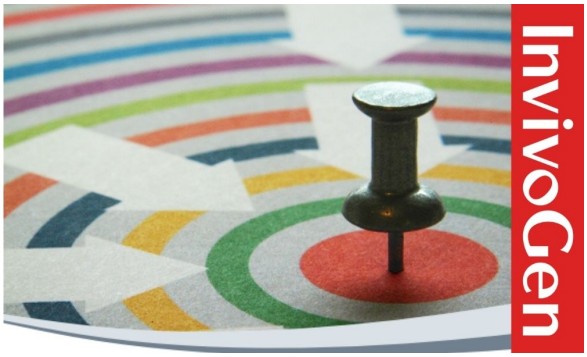

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A T CELL EPITOPE IN VP1 OF FOOT-AND-MOUTH DISEASE VIRUS IS IMMUNODOMINANT FOR VACCINATED CATTLE

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Synthetic peptides representing regions of the VP1 protein of foot-and-mouth disease virus strain O1 Kaufbeuren were screened for their ability to stimulate proliferation of PBMC from virus vaccinated cattle. Sites were identified at residue 21-40 (peptide FMDV32) and in the region C-terminal to residue 161. Cells responding to FMDV32 were MHC class II-restricted, CD4+ and secreted IL-2. Thus, this region is defined as a Th site. Of 19 virus vaccinated Friesian cattle, 89% (17/19) responded to purified virus while 37% (7/19; 41% of virus responders) also responded to FMDV32 suggesting that this site is immunodominant for the cattle used. Furthermore, immunisation of FMDV32 responder and non-responder cattle with a related peptide, FMDV5 (FMDV32 co-linearly synthesized with the 141-160 VP1 B cell site), induced neutralizing antibody and a virus-specific T cell population in the FMDV32-responder but not the non-responder animals.

Foot-and-mouth is a highly contagious virus disease of cloven hooved animals and is a major threat to the livestock industry. The causative agent of foot-and-mouth disease is aphthovirus or, more commonly, FMDV.[2] The infectious virus particle is a single strand, positive sense RNA genome surrounded by a protein capsid comprising 60 copies each of four virus coded polypeptides (VP1-4). The structural proteins VP1-3 are known to contribute to the antigenicity of the intact virus particle although only VP1 has been shown to induce neutralizing antibody in isolation (1, 2). In the case of vaccinated cattle, protection has been correlated with neutralizing antibody titer (3, 4).

The disease is controlled by a variety of measures that include the slaughter of virus-infected and -exposed animals and/or vaccination of susceptible livestock. Current vaccines against FMDV are produced from chemically inactivated virus and have a number of problems associated with their stability and innocuity; not least is the concern that these vaccines have been the source of some of the recent outbreaks in Europe. Consequently, much effort is being focused on the development of synthetic Ag vaccines.

Recombinant VP1 prepared in *Escherichia coli* has been shown to protect both pigs and cattle (5). However, the immunity induced was inferior to inactivated virus and required approximately 1000-fold higher dose of the immunogen to induce comparable protection to that of conventional vaccines (6). After observations by Strohmaier et al. (7), the VP1 regions 146-154 and 200-213 were confirmed as epitopes for neutralizing antibody by the use of synthetic peptide immunogens (8, 9). Subsequently, synthetic peptides based on 141-160 of O1K, with or without carrier proteins, have been used to raise antibodies in mice, rabbits, and guinea pigs and to protect the latter from challenge against homologous virus (10–12). Furthermore, the immunogenicity of this peptide was increased when fused with hepatitis B core protein (13). However, the natural extension of these results to protect the major target species, i.e., cattle, has only been reported by DiMarchi et al. (14) using a synthetic peptide that contained residues 141-158 and 200-213 of O1K linked by a diproline-serine spacer.

The use of a carrier protein such as keyhole-limpet hemocyanin or hepatitis B cores to potentiate the response to a synthetic peptide detracts from the advantages of a defined peptide vaccine. In particular, boosting of the peptide-specific response may be limited by the genetic restriction of carrier-specific Th cells. In mice, the antibody response to the carrier-free synthetic peptide 141-160-cys is restricted by the major histocompatibility complex in the context of H-2^k (15) and can be overcome by the addition of "foreign" Th cell determinants. We have previously reported that the murine antibody response to FMDV is T cell dependent (16) and that, in contrast to the serotype-specificity of the antibody response, bovine T cells are serotypically cross-reactive (17). Thus, native FMDV T cell determinants should suffice to improve the immunogenicity of a synthetic peptide Ag based on 141-160.

Epitopes recognized by T cells can be determined either by assessing the ability of isolated proteins or peptides to stimulate virus-specific T cells, or, by assessing the ability of isolated proteins or peptides to induce a T cell population that recognizes the native proteins. Both approaches have been used and in this report we present evidence for a T cell epitope located in VP1 that is recognized by class II restricted, CD4+ T cells in a high proportion of the vaccinated cattle examined. This epi-

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[2] Abbreviations used in this paper: FMDV, foot-and-mouth disease virus; O1K, FMDV strain O1 Kaufbeuren; O1BFS, FMDV strain O1 British Field strain 1860; O1M, FMDV strain O1 Manisa; ACruz, FMDV strain A24 Cruzeiro; ASau, FMDV strain A Saudi 23/86; A22, FMDV strain A22 Iraq 24/64; Asia, FMDV strain ASIA1 India; C1, FMDV strain C1 Noville; hrIL-2, human recombinant interleukin-2.

tope is distinct from the previously described site at VP1:150-160 and, in appropriate cattle, can induce T cells that respond to virus.

MATERIALS AND METHODS

Peptides

Peptides were prepared by the solid-phase synthesis method of Merrifield et al. (18) and correspond to VP1 sequences of the FMDV strain O1K (type O, subtype 1, strain Kaufbeuren). Peptides that combine discontinuous regions of the protein (e.g., FMDV4) were prepared in a single synthesis.

Set 1. FMDV4, (1-20)(141-160); FMDV5, (21-40)(141-160); FMDV6, (37-56)(141-160); FMDV7, (57-76)(141-160); FMDV8, (77-96)(141-160), FMDV9, (97-116)(141-160); FMDV10, (121-160); FMDV16, (134-160); FMDV24, (141-160)-CG.

Set 2. FMDV11, (140-179); FMDV12, (141-160)(181-200); FMDV30 (134-213); FMDV29, (161-213); FMDV28, (161-180)(196-213); FMDV26, (200-213).

Set 3. FMDV31, (31-40); FMDV32, (21-40), FMDV33, (26-35), FMDV34, (21-30).

mAb

Ascitic fluid from the murine anti-bovine hybridomas IL-A12 (anti-CD4), IL-A17 (anti-CD8), IL-A19 (monomorphic anti-class I), and IL-A21 (monomorphic anti-class II) were kindly provided by Dr. I. Morrison of the International Laboratory for Research into Animal Diseases, Nairobi, Kenya.

Animals

Friesian cattle were immunized against FMDV by inoculation of a standard cattle dose of commercial vaccine (a gift from Coopers Animal Health, Pirbright, Surrey, England). Five cattle were immunized against O1BFS alone; eight cattle were immunized against O1BFS of which four were subsequently vaccinated with ASau and the other four with Asia; two cattle were immunized trivalently against O1M, A22, and C1; two cattle were immunized bivalently against O1BFS and A22; one animal was immunized bivalently against A22 and C1 and one animal was immunized against A24 (type A, subtype 24, strain Cruzeiro). Another two cattle were each inoculated with 0.5 mg FMDV5 in 2 ml of 50% IFA.

PBMC

Blood was collected into heparin and used immediately for the preparation of PBMC. Buffy coat cells were diluted with cold HBSS and the PBMC isolated by floatation over Lymphopaque (p = 1.086; Nycomed, Sweden)(800 × g, 45 min). Cells at the gradient interface were aspirated, the contaminating E lysed with ice cold ammonium chloride (0.83% w/v) and the resulting mononuclear cell suspension washed with HBSS. The cells were held on ice until required.

Accessory Cells

Autologous irradiated (2000 rad) PBMC were used as a source of APC.

T Cell Lymphoblasts

PBMC were cultured at 2×10^5 cells/well with an optimal concentration of purified O1BFS. After 7 days the viable cells were collected over Lymphopaque (400 × g, 20 min) and diluted to 1×10^5/ml in complete medium containing 10 IU/ml hrIL-2 (Amgen Biologicals, Amersham International plc, England). The cells were fed with IL-2 at 3- to 4-day intervals and used in proliferation assays after 7 to 10 days.

In Vitro Immunization with Peptide

PBMC were in vitro immunized against FMDV5 by culturing 0.5 ml of complete RPMI containing 2×10^6 cell and various concentrations of peptide in a 24-well cluster plate (Costar Mark II, catalogue no. 3424). After 4 days, 0.5 ml of complete RPMI was added to each well and, after a further 4 days, 0.5 ml of complete RPMI containing 15 IU/ml hrIL-2. On day 11, the contents of each well was split into two wells and 0.75 ml of fresh medium containing 10 IU/ml hrIL-2 was added. Fourteen days after the initiation of cultures the viable cells were collected over Lymphopaque (400 × g, 20 min) and were tested for enhanced proliferation in response to FMDV5 compared to cells cultured without peptide or immunized with an irrelevant peptide.

Proliferation Assay

Proliferation of PBMC was determined as previously described (17). Two × 10^5 PBMC/well were suspended in 0.2 ml RPMI 1640 medium containing 5% (v/v) heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 0.05 mM 2-ME, antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and various concentrations of purified O1BFS virus (19) or peptide. Cells were cultured in 96-well, round-bottom plates at 37°C for 5 days and 0.2 μCi [^{3}H]TdR/well was added over the last 16 h of culture. Cells were harvested using a Multimash (Dynatech Ltd.). T lymphoblasts (2.5×10^4/well) or in vitro immunized cells (5×10^4/well) were stimulated with virus or appropriate peptides in the presence of 2×10^5 accessory cells for 4 or 5 days, respectively. Proliferation, as correlated with the incorporation of label, was determined by liquid scintillation spectroscopy and expressed as Δ-cpm (cpm of test sample − cpm of medium alone) for the arithmetic mean of triplicate cultures. Proliferation was considered positive if the mean Δ-cpm for a test sample exceeded three times the SE obtained with medium alone and the response was dose dependent.

Sequence Analysis

The sequence of VP1 of FMDV O1K and O1BFS was examined by the following methods.

Motif. Motifs (20) (charged residues or glycine followed by two or three hydrophobic and then polar residues or glycine) were determined where D, E, H, K, R, and G were taken as charged residues, A, V, L, I, F, M, W, T, and Y (or P if it is in the fourth position of a five residue motif) as hydrophobic residues and D, E, H, K, R, N, Q, S, T, and Y as polar/charged residues. Attention was focused on motifs that conformed to the modification of Rothbard and Taylor (21) (eight residues centred around a motif such that one of the terminal residues is charged and the other terminus and the two central residues are hydrophobic).

AMPHI. Sites of hydrophobic variation consistent with the formation of an amphipathic helix were determined according to the algorithm developed by Berzofsky and colleagues (22). Computation used Fauchere-Pliska (23) hydrophobicity scales of amino acids and a block length of 11 with amphipathic score threshold set at 8.

PREDICT. A secondary structure prediction suite developed by Eliopoulis et al. (24) was used to determine regions of α-helix, β-sheet, and random turns or coils as a consensus (joint) of a number of well known methods that include those of Burgess et al., Dufton and Hider, Chou and Fasman, Garnier et al., Kabat and Wu, Nagano, McLaughlan and Lim.

RESULTS

Synthetic peptides. Figure 1 shows the peptides used in this study and their relationship to the mature VP1 polypeptide. The peptides were divided into three sets: set 1 contains peptides representing sequences N-terminal to and including 141-160 of VP1 and set 2 contains

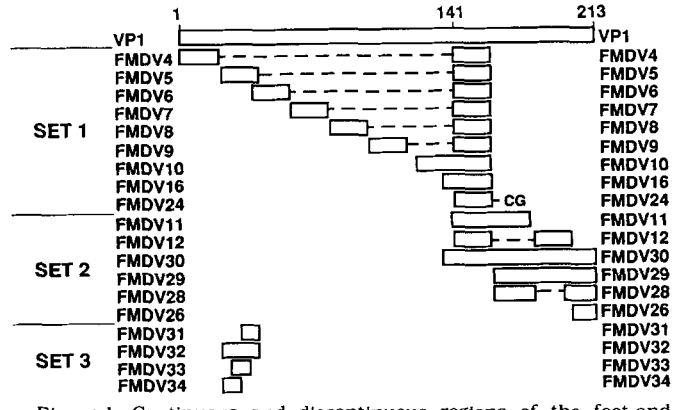

Figure 1. Continuous and discontinuous regions of the foot-and-mouth disease virus protein VP1 were synthesised as co-linear peptides. The VP1 regions defined by the peptides are shown above. The precise limits of each region are detailed in *Materials and Methods.*

peptides representing sequences C-terminal to 134 or 141 of VP1. To further define a region of interest, set 3 was synthesized to contain four overlapping peptides which span the region 21-40.

Screening of synthetic peptides. The peptides and purified virus were screened for their ability to stimulate PBMC to proliferate. Figure 2 shows representative titrations for three O1BFS vaccinated cattle determined at 36 mo after revaccination.

Figure 2*a* shows the response of animal PR90 stimulated with purified virus. Proliferation was found to peak at a concentration of 3.16 nM with respect to the structural proteins. The molarity of structural proteins was calculated on the assumption that the virus composition is 30% ssRNA, 21% for each of VP1, 2, and 3 and 7% VP4 by weight where VP1, 2, and 3 have m.w. of approximately 24,000 and VP4 has a m.w. of approximately 8500. The response to medium alone was 16972 cpm and the variation between replicates was 18%. The responses to peptide set 1 and set 2 are shown in Figure 2 *b* and *c*, respectively. Peptides FMDV6, FMDV7, FMDV9, FMDV10, FMDV11, FMDV28, FMDV29, and FMDV30 were stimulatory (as defined in *Materials and Methods*) at concentrations in excess of 1 μM and the strongest response was elicited by peptide FMDV30. A weak and inconsistent response against FMDV24 was detected for up to 6 mo whereas response to peptides FMDV6, FMDV7, FMDV9, FMDV10, FMDV11, FMDV28, FMDV29, and FMDV30 persisted for at least 36 mo.

Figures 2 *d* to *f* show the responses obtained for animal PR91. The peak of stimulation by virus occurred at 3.16 nM (Fig. 2*d*) and both the background response (3823 cpm) and the variation between replicates (8%) were reproducibly low. Peptides FMDV6, FMDV9, FMDV10 (Fig. 2*e*), and FMDV12 (Fig. 2*f*) were stimulatory at concentrations in excess of 316 nM. Response against FMDV24 was detected for 3 to 4 mo although this required in excess of 3.16 μM peptide and, as for PR90, was inconsistent between assays. In contrast, FMDV5 (Fig. 2*e*), FMDV29, and FMDV30 (Fig. 2*f*) elicited a strong response which was detected for over 36 mo. The response induced by FMDV29 and FMDV30 could not be distinguished over the concentration range used (1 nM to 3.16 μM). FMDV5 gave a maximal response over the range 10 to 100 nM.

Figures 2 *g* to *i* show the response of animal PR92. Peak stimulation with virus was observed at 31.6 nM (Fig. 2*g*) and, whereas, the response to medium alone (16326 cpm) was high, the variation between replicates (11%) was intermediate between that of PR90 and PR91. Peptides FMDV5, FMDV6, FMDV7, FMDV10 had stimulatory activity for up to 6 mo and FMDV24 was weakly stimulatory for up to 4 mo after revaccination. Only FMDV9 (Fig. 2*h*) was strongly stimulatory at 36 mo.

Analysis of response to FMDV5. Peptide FMDV5 is a hybrid of two discontinuous regions of VP1, namely 21-40 and 141-160. The peptides in set 3 were synthesized to examine the contribution of each region.

Figure 3 shows the responses of PR90, PR91, and PR92 to FMDV5, FMDV24, and FMDV31-34. Only PR91 responded strongly to FMDV5 (Fig. 3*b*). In this animal, the response to FMDV5 and FMDV32 could not be distinguished at equimolar concentrations. Peptide FMDV31 was also stimulatory at concentrations in excess of 100 nM. Animal PR90 responded to FMDV32 at concentrations equal to or more than 316 nM whereas no response was detected for FMDV5. FMDV24 did not induce a response in any of the animals and no other responses were significant.

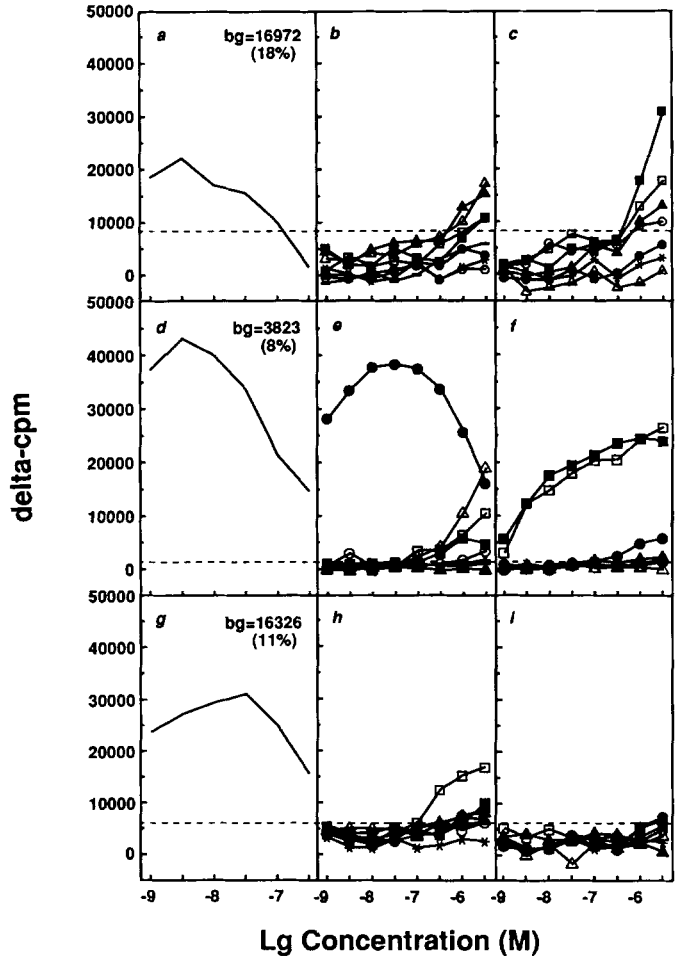

Figure 2. Proliferation of PBMC from animals PR90 (*a* to *c*), PR91 (*d* to *f*), and PR92 (*g* to *i*) in response to virus and synthetic peptides. PBMC (2×10^5) were cultured with various concentrations of virus (*a, d, g*), set 1 peptides (*b, e, h*), or set 2 peptides (*c, f, i*) and proliferation was determined by [^{3}H]TdR incorporation after 5 days. Proliferation is expressed as Δ-cpm. *bg*, background for medium alone + percentage SE in parentheses. *b, e, h*, ○, FMDV4; ●, FMDV5; △, FMDV6; ▲, FMDV7; –, FMDV8; □, FMDV9; ■, FMDV10; *, FMDV24; *c, f, i*, ○, FMDV11; ●, FMDV12; *, FMDV24; △, FMDV26; ▲, FMDV28; □, FMDV29; ■, FMDV30. ---, Three times the background SE.

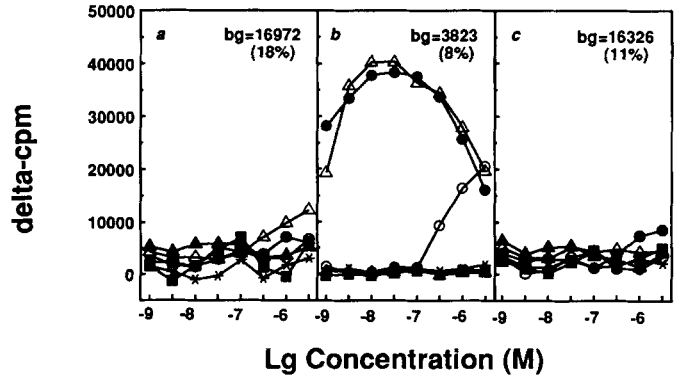

Figure 3. Proliferation of PBMC from three FMDV vaccinated cattle in response to set 3 synthetic peptides defining the region 21-40 of protein VP1. Experimental conditions and expression of data as for Figure 2. *a*, PR90; *b*, PR91; *c*, PR92. ●, FMDV5; *, FMDV24; ○, FMDV31; △, FMDV32; ▲, FMDV33; ■, FMDV34.

Evidence that FMDV32 Site is Th epitope. mAb against bovine CD4, CD8, MHC class I, and MHC class II were used to determine the phenotype and the MHC restriction of cells responding to FMDV32. The proliferation of virus or peptide stimulated T lymphoblasts was inhibited by anti-CD4 and anti-class II but not by anti-CD8 or anti-class I antibodies (Table I). In addition, supernatant harvested from cultures 24 h after stimulation was able to support the growth of an IL-2-dependent cell line, TC-6 (25) (data not shown).

Dominance of response to FMDV32. To examine whether the response of animal PR91 to FMDV32 was atypical, peptide recognition was tested in a greater number of vaccinated cattle. Table II shows the ability of FMDV32 to stimulate PBMC from cattle vaccinated by a variety of regimes against different strains of FMDV.

Of the 19 animals examined, the majority (17/19) had not been vaccinated within at least 6 mo before assay. A total of 17 of the 19 cattle (89%) gave a significant response against virus and seven (37%) against FMDV32. All of the peptide responders were also virus responders.

TABLE I

Inhibition of virus and FMDV32-induced proliferation by mAb[a]

Treatment Of Cells with	PBMC Stimulated with (Δ-cpm)[b]		
	Medium	Virus	FMDV32
Control Ig	2,833	41,322	37,915
Anti-CD4		12,311 (70.2)	6,203 (83.6)
Anti-CD8		37,341 (9.6)	37,414 (1.3)
Anti-class I		43,901 (−6.2)	40,110 (−5.79)
Anti-class II		6,011 (85.4)	4,322 (88.6)

[a] T lymphoblasts from animal PR91 (2.5×10^4/well) were stimulated in vitro with virus (3.16 nM) or peptide FMDV32 (10 nM) in the presence of accessory cells (2×10^5/well). Various mAb were added at 10-times the concentration required for saturation of Ag (as determined by flow microfluorimetry analysis) at the initiation of the cultures. Proliferation was determined by the incorporation of [methyl-^{3}H]TdR over the last 16 h of a 96-h culture. Anti-CD4, -class I, and -class II antibodies were mouse IgG2a, anti-CD8 was mouse IgG1 and the control Ig was mouse IgG2 (20 μg/ml; Becton Dickinson UK Ltd).

[b] Stimulation is expressed as Δ-cpm. Numbers in parentheses indicate the percent inhibition calculated as ((Δ-cpm control Ig − Δ-cpm test Ig)/Δ-cpm control Ig) × 100.

TABLE II

Virus and FMDV32-induced proliferation in once, twice, and thrice vaccinated cattle[a]

Vaccination Status[b]	Ag[c]	Response to	
		Virus	FMDV32
Primary	O1BFS	2/2	1/2
	O1, A22	2/2	0/2
	O1M, A22, C1	2/2	1/2
	A22, C1	0/1	0/1
Secondary	O1BFS, ASau	3/4	1/4
	O1BFS, Asia	4/4	3/4
Tertiary	O1	3/3	1/3
	A24	1/1	0/1
Total		17/19	7/19
% Responders[d]		89.5	36.8
% Specificity[e]		100.0	41.2

[a] PBMC from FMDV vaccinated cattle were stimulated with virus (1 to 316 nM) or peptide (1 nM to 3.16 μM). Proliferation was determined by the incorporation of [methyl-^{3}H]TdR over the last 16 h of a 5 day culture. Responsiveness to Ag was determined as defined in *Materials and Methods*.

[b] Vaccination status: primary, once vaccinated; secondary, twice vaccinated (these animals immunized against O1BFS and followed by either ASau or Asia); tertiary, thrice vaccinated.

[c] Strains of virus used in vaccine as defined in *Materials and Methods*.

[d] Percent responders = (no. of animals responding to Ag/total number of animals) × 100.

[e] Percent specificity = (no. of animals responding to virus or peptide/no. of animals responding to virus) × 100.

Thus, the proportion of cattle responding to peptide compared to the proportion responding to virus was 7/17 (41%) and all responding cattle did so at 10 to 1000 nM. However, the majority of the cattle responding to FMDV32 responded maximally at 100 nM or more, requiring a 10-fold higher dose compared to PR91. Similar results were obtained with FMDV5 (data not shown).

Immunogenicity of FMDV5 in cattle. The high proportion of virus vaccinated cattle which responded to FMDV5 and FMDV32 together with the Th activity of FMDV32 in animal PR91 led us to consider whether FMDV5 could induce virus-specific T cells.

Several FMDV naive cattle were tested for their ability to respond to FMDV5 by in vitro priming of lymphocytes (see *Materials and Methods*). Four cattle were selected and paired such that each pair comprised one peptide responder and one peptide non-responder. One pair was vaccinated with commercial O1BFS virus vaccine and the other with FMDV5 and the proliferative response of PBMC determined. At 21 days post-vaccination, the animals were challenged intradermalingually and observed for protection.

Both of the animals immunized with virus vaccine were protected against challenge (Table III) and developed a T cell response against virus (Fig. 4 *a* and *b*) whereas only

TABLE III

Response of responder and non-responder cattle to vaccination with virus or peptide FMDV5[a]

Ear Tag	Vaccinated with[b]	SNT[c]	Protection[d]
RD56 (R)[e]	O1BFS	355	P
RD57 (NR)	O1BFS	178	P
RD58 (R)	FMDV5	178	P
RD59 (NR)	FMDV5	8	NP

[a] Cattle were selected for response to FMDV5 by in vitro immunization and paired one non-responder with one responder. Each pair was vaccinated and 21 days after vaccination the animals were challenged with 100,000 50% tongue infectious units of virus by intradermalingual inoculation.

[b] O1BFS commercial vaccine, 2 ml dose; peptide FMDV5 emulsified in IFA, 2 ml dose (250 μg/ml).

[c] Reciprocal of the virus neutralizing titer of serum at day 21 after vaccination.

[d] P = protected; NP = not protected.

[e] R = FMDV5 responder; NR = FMDV5 non-responder.

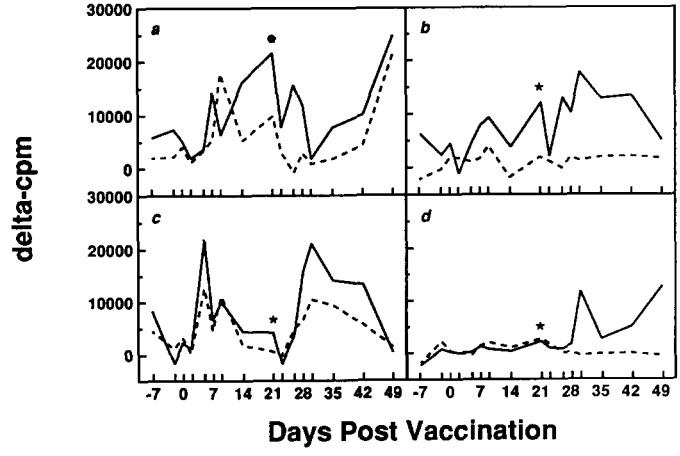

Figure 4. Proliferation of PBMC from peptide responder (RD56 and RD58) and non-responder (RD57 and RD59) vaccinated cattle stimulated with virus or peptide FMDV5. PBMC (2×10^5) were cultured with virus (equivalent to 10 nM VP1) or FMDV5 (10 nM) and proliferation was determined by [^{3}H]TdR incorporation after 5 days. *a*, RD56 virus vaccinate; *b*, RD57 virus vaccinate; *c*, RD58 peptide vaccinate; *d*, RD59 peptide vaccinate. ——, Stimulation with virus; – – –, stimulation with peptide. *, challenge with 100,000 50% tongue infectious units of O1K virus.

the responder developed a T cell response against FMDV5 (Fig. 4*a*). In the pair of animals immunized with peptide, only the responder was protected against challenge (Table III) and developed a T cell response to both peptide and virus before challenge (Fig. 4*c*). A proliferative response to FMDV24, that is the 141-160 component of FMDV5, was not detected during the period of assay although the two virus vaccinated cattle and the FMDV5 vaccinated peptide responder did develop neutralizing antibody (Table III) and antibody against 141-160 as determined by ELISA (data not shown). In all cases, infection resulted in either restimulation or induction of an anti-virus T cell response.

Prediction of T cell sites and concordance with stimulatory activity of peptides. The VP1 sequence of FMDV O1K was analyzed for the presence of putative T cell sites according to the algorithms MOTIF and AMPHI. Fourteen sites were identified by MOTIF (20), of which 2 complied with the more stringent constraints of Rothbard and Taylor (21), and 11 by AMPHI. Of the latter, seven had periodicity consistent with that of an α-helix (average periodicity 100°), two were consistent with 3/10-helices (average periodicity 120°), and two had periodicity ranging from 80 to 130°. The regions 37-67 and 153-192 contained clustered predictions from both algorithms (amphipathic blocks 37-44, 46-55, 57-60, and 62-67 overlapped with five Rothbard patterns; amphipathic blocks 153-160, 169-178, and 189-192 overlapped with six Rothbard patterns) although neither of the regions contained a Rothbard and Taylor MOTIF. The region 37-67 was represented in the peptides FMDV6 and FMDV7 and peptides FMDV11, FMDV12, FMDV16, FMDV24, FMDV28, FMDV29, and FMDV30 contained all or parts of the predicted sites in the region 153-192. The amphipathic block at 153-160 was present in all peptides except FMDV26, FMDV28, FMDV29, and the set 3 peptides. Amphipathic sites at 94-106 and 109-111 and 121-130 were represented in FMDV9 and FMDV10, respectively, and both peptides contained Rothbard patterns. The Rothbard pattern in FMDV10 was one of the two Rothbard and Taylor patterns. The stimulatory activity of the FMDV peptides over a 3-y period is summarized in Table 4. In general, the peptides representing clusters of predictions had stimulatory activity at concentrations of 1 μM or greater. No stimulatory activity was detected for the strongly predicted α-amphipathic site at residues 6-18 (contained in FMDV4). Conversely, the most strongly stimulatory peptide (FMDV5) had no prediction by either MOTIF or AMPHI. Peptide FMDV31 contained most of the activity of FMDV5 and FMDV32 (Fig. 3*b*). This region was an α-helix by the JOINT algorithm but could not be clearly distinguished from the β-sheet predictions in the same region.

TABLE IV

Summary of stimulatory activity of FMDV peptides[a]

Peptide	Sequence	Stimulatory Activity[b]	Predicted Activity[c]
FMDV4	[1–20][141–160]	–	+
FMDV5	[21–40][141–160]	++++++	–
FMDV6	[37–56][141–160]	+++	+
FMDV7	[57–76][141–160]	+++	+
FMDV8	[77–96][141–160]	–	+
FMDV9	[97–116][141–160]	+++	+
FMDV10	[121–160]	++	+
FMDV11	[140–179]	+++	+
FMDV12	[141–160][181–200]	+++	+
FMDV16	[134–160]	+	+
FMDV24	[141–160]CG	+	+
FMDV26	[200–213]	–	–
FMDV28	[161–180][196–213]	+++	+
FMDV29	[161–213]	+++++	+
FMDV30	[134–213]	+++++	+
FMDV31	[31–40]	++++	+
FMDV32	[21–40]	++++++	–
FMDV33	[26–35]	–	–
FMDV34	[21–30]	+	–

[a] PBMC from FMDV vaccinated cattle were stimulated with peptide (1 nM to 3.16 μM) and proliferation was determined by the incorporation of [^{3}H]TdR over the last 16 h of a 5-day culture. The significance of the response was determined as defined in *Materials and Methods*.

[b] Subjective reactivity of peptides: (–), not stimulatory; (+), activity not always detected; (++) weakly stimulatory at 1 μM; (+++), moderately stimulatory at 1 μM; (++++), strongly stimulatory at 1 μM; (+++++), strongly stimulatory at 100 nM; (++++++), strongly stimulatory at 10 nM.

[c] Sequence contains either a MOTIF (20, 21) or an amphipathic (22) T cell Ag prediction or both.

DISCUSSION

In this report we have examined the specificity of bovine T cells with respect to peptides representing the structural protein VP1 of FMDV, strain O1K.

In the first experiment, the ability of purified O1BFS virus and synthetic O1K peptides to stimulate PBMC from three cattle vaccinated with O1BFS was examined. Although O1K and O1BFS are different strains of FMDV, the VP1 protein is conserved at 210 of the 213 residues and only one of the substitutions is non-conservative (26–28). Stimulation was defined as significant if it induced a dose-dependent proliferation that exceeded 3 × SE of the background for any given animal. This distinction was necessary since one of the animals (PR90) readily responded to IL-2, without prior stimulation by antigen or lectin, giving rise to a high background and low specific stimulation (Ag-induced response/background response).

The optimum stimulatory dose of virus for two of the animals, PR90 and PR91, was 3.16 nM (361 ng/ml 146S virus) with respect to the structural proteins VP1-4. The third animal required 31.6 nM. In contrast, the majority of the synthetic peptides required 30 to 300-fold excess ($\geq$1 μM) over the virus equivalent. The peptides FMDV5, FMDV29, and FMDV30 were of particular interest because they induced a response with similar magnitude and dose to that of virus. The response to FMDV5 was almost identical to that of virus albeit in one animal only (PR91). Because peptide FMDV5 is a hybrid of the VP1 regions 21-40 and 141-160, the lack of response to 141-160 (FMDV24) and some other hybrid peptides such as FMDV4 and FMDV8 suggested that the activity of FMDV5 was not due to the 141-160 component. To address the possibility that the 141-160 component was influencing the antigenicity of FMDV5, peptides were synthesized across the region 21-40 and their stimulatory activity examined.

At equimolar concentrations, the responses to FMDV5 and FMDV32 (Fig. 3*b*) were indistinguishable, suggesting that the activity of FMDV5 was entirely due to the region 21-40. Moreover, responses to the overlapping peptides FMDV31, FMDV33, and FMDV34 (Fig. 3*b*) indicated that the epitope included residues 31-40 (FMDV31). The observation that FMDV31 required around 1000-fold more peptide than FMDV32 for an equivalent stimulation is consistent with FMDV31 being short of the minimal stimulatory sequence for maximum stimulation as re-

ported by Rothbard et al. (29) who have demonstrated that the sequential removal of residues from the minimal sequence first increases the dose required for maximum stimulation and then decreases the stimulatory activity.

The inhibition of virus- or peptide-induced proliferation by mAb identified the phenotype of FMDV32-responding cells as $CD4^+$ and MHC class II restricted. Furthermore, stimulation resulted in the production of IL-2 and, thus, the region 21-40 is functionally defined as a Th cell site. The FMDV32-specific responses of a larger sample of virus vaccinated cattle (7/19 total; 7/17 virus responder animals) suggests that this site is dominantly recognized in the breed used (Friesian; *Bos taurus*). The MHC class II haplotype of our cattle is unknown and experiments are currently in progress to determine the restricting element for the FMDV32 epitope. Relatively little is known about the class II molecules in cattle although it is generally believed that the selective breeding practices used in animal husbandry could skew the distribution of MHC alleles in any group of animals. Given also that the expression of the T cell repertoire is influenced both at the level of processing and competition between processed Ag fragments for association with a particular MHC molecule (30–32), it will be important to establish whether FMDV32 is immunodominant for other breeds of cattle.

Having identified a putative Th cell site we then tested it for the ability to induce a population of T cells capable of recognizing the native protein. Four cattle were immunized with virus or peptide and their responses to both Ag were determined. Only the animals predetermined to be responders to FMDV5 responded after immunization with virus or peptide. The virus vaccinated cattle and the peptide immunized responder were protected when challenged with O1K virus and the neutralizing antibody titers of the protected cattle were in excess of the threshold determined for the protection of O1BFS vaccinated cattle challenged with homologous virus (4; 1/138 for 50% protection). Moreover, the neutralizing antibody titer induced in the peptide immunized responder was as high as that induced by virus in one of the virus vaccinated animals. All of the protected animals developed IgG1 and IgG2 before challenge although the peptide vaccinate developed predominantly IgG1 (T. Collen, unpublished observations). In contrast, the peptide immunized nonresponder had a neutralizing antibody titer of only 1/8 and no IgG. Immunization with the VP1 sequence 141-158 linked to 200-213 has previously been shown to protect cattle using 5 mg of peptide in CFA or 1 mg in CFA followed by 0.2 mg in IFA (14). In the current experiment, protection was achieved after a single dose of 0.5 mg of FMDV5 in IFA. Despite the small number of animals involved, the efficacy of a lower dose of Ag and the correlation between T cell responsiveness, sero-conversion, and protection emphasizes the importance of T cell epitopes in new generation vaccines.

The failure of the peptide immunized animals to develop a proliferative response to 141-160, one animal of which also lacked a 141-160-specific antibody response, suggests that the 141-160 region has no intrinsic helper activity in cattle. This is in contrast to experimental data from guinea pigs (11) and may be due either to 1) the absence of a non-native, C-terminal cysteine in our peptide, because this was required to confer immunogenicity on free 141-160 peptide (11), or 2) MHC restriction as described in mice (15). The responses of the FMDV5 immunized responder to virus and peptide were similar at each point of assay (Fig. 4*c*) whereas the responses of the virus immunized responder (Fig. 4*a*) were biphasic and indicates the mono-specific nature of the T cells primed by peptide compared to virus.

Sercarz et al. (33) have suggested that the antibody response after priming with a macromolecule or peptide is dependent upon the regulatory T cell determinant(s) and the B cell determinant being in close proximity on a single processed antigenic fragment. If extrapolated to FMDV, then FMDV32-reactive T cells might be expected to help B cells that recognize an epitope involving residues 43, 44, and 45 (34) (antibody site 3) rather than the 141-160 epitope (34) (antibody site 1). Nevertheless, the ability of the FMDV32 reactive T cells to help 141-160-specific B cells after immunization with only those sites is encouraging for use of sequences from other more conserved virus proteins, such as the polymerase or VP4, as the T cell epitope(s) of future vaccines. Clearly the efficacy of FMDV5 has to be tested in a much larger group of animals.

Two other peptides were found to be strongly stimulatory: These were the peptides FMDV29 (161-213) and FMDV30 (134-213). For the animal PR91, response to a related peptide suggested that the site recognized by this animal involves the region 181-200 (peptide FMDV12; Fig. 2*f*). It is unlikely that the response was influenced by the 141-160 region because the response to FMDV29 and FMDV30 was identical at equimolar concentrations and no response was detected to FMDV24. In contrast, animal PR90 was stimulated more effectively by FMDV30 than FMDV29 suggesting that residues within the 141-160 region might influence recognition. However, responses to peptides FMDV11 (140-179) and FMDV28 (161-180, 196-213) favored the involvement of 161-180 because these peptides had similar stimulatory activity. Proliferative response to FMDV26 (200-213) was never detected in any animal tested although weak responses to all or some of peptides FMDV6, FMDV7, FMDV9, and FMDV10 were detected for each of the animals.

Most of the peptide sets used in this study did not include overlapping sequences and were not synthesized with the determination of T cell epitopes in mind. However, a number of the peptides contained, or overlapped with, putative T cell sites as determined by predictive algorithms. In general, stimulatory activity was associated with peptides that represented regions where both MOTIF and AMPHI sites clustered. The activity identified for 21-40 was not predicted by MOTIF or AMPHI, although the region was predicted to be α-helical by JOINT and can be seen to adopt helical conformation as it passes through the capsid of the virus (D. Stuart and E. Fry, personal communication).

In conclusion, we have demonstrated a T cell site at 21-40 of O1K FMDV VP1 that is distinct from 141-160 and which is recognized by a high proportion of vaccinated Friesian cattle. This epitope can provide B cell help when linked to the 141-160 site. Currently we are addressing the fine specificity of this epitope and the MHC haplotype against which the epitope is recognized. Further experiments are in progress to assess regions of the

structural and non-structural proteins for the presence of serotypically conserved epitopes.

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