

T cell-stimulatory fragments of foot-and-mouth disease virus released by mild treatment with cathepsin D

Marie-José C. van Lierop,¹ Johannes M. van Noort,² Josée P. A. Wagenaar,¹ Victor P. M. G. Rutten,¹ Jan Langeveld,³ Rob H. Melen³ and Evert J. Hensen^{1*}

¹Department of Immunology, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, P.O. Box 80.165, 3508 TD Utrecht, ²TNO Medical Biological Laboratory, P.O. Box 5815, 2280 HV, Rijswijk and ³Institute of Animal Science and Health, ID-DLO, P.O. Box 65, 8200 AB, Lelystad, The Netherlands

Cathepsin D and cathepsin B are endosomal/lysosomal proteases that are thought to play a role during *in vivo* antigen processing, releasing fragments for binding to major histocompatibility complex class II products and subsequent presentation to T cells. Here we treated purified foot-and-mouth disease virus (FMDV) strain A₁₀Holland with both enzymes. Cathepsin D, but not cathepsin B, was shown to release fragments from reduced or non-reduced FMDV under mild conditions *in vitro*. Twenty-eight predominant cathepsin D-released fragments were purified by HPLC and identified by amino acid composition analysis and sequencing. The unseparated set of fragments produced (the digest) was able to stimulate T cells from eight vaccinated cattle. With respect to the response to intact virus the extent of the response to the digest differed between animals: four animals could be classified as good responders, three as intermediate responders and one as a low responder.

Subsequently, we investigated the proliferative T cell response to a large set of synthetic peptides in detail for two animals, one belonging to the group of good responders, the other being the low responder. The peptides covered all 28 cathepsin D-released fragments analysed and also several sequences not recovered from the digest. In this way seven T cell sites could be identified, five of which coincided with cathepsin D-released fragments. The other two T cell sites were VP2[54–72], being a homologue of a T cell site identified for FMDV strain O₁K and the N terminus of VP4. Whether the most dominantly recognized T cell site was recovered from the digest or not was shown to be related to the good or low response to the digest. These findings suggest a role for cathepsin D in the release of some but not all T cell-stimulatory fragments from FMDV.

Introduction

CD4-positive T cells generally respond to antigenic peptides physically associated with major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells (APC) (Schwartz, 1985). The generation of this MHC class II/peptide complex requires a process of internalization and proteolytic degradation of the antigen and binding of its fragments to MHC class II products (i.e. antigen processing) (Brodsky & Guagliardi, 1991). The sub-cellular compartments in which these processes take place and how they take place has not yet been fully established. Endosomes, lysosomes and MHC class II-specific vesicles have all been suggested to be locations where proteolytic enzymes might operate and/or where antigenic fragments might bind to MHC class II products (Guagliardi *et al.*, 1990; Peters *et al.*, 1991). Most studies

to characterize the proteases playing a role in antigen processing have involved the use of specific inhibitors of different classes of acidic proteases (Puri & Factorovitch, 1988; Vidard *et al.*, 1991, 1992; Bennett *et al.*, 1992). More direct evidence that an antigen can be processed into T cell-stimulatory fragments by a certain enzyme can be provided by *in vitro* digestion and subsequent presentation of the digest to specific T cells. Both types of studies have implicated thiol proteases such as cathepsin B and aspartyl proteases such as cathepsin D and E as being involved in antigen processing (Diment, 1990; Bennett *et al.*, 1992; Van Noort *et al.*, 1991). Special attention has been focused upon cathepsin D and cathepsin B since these are by far the predominant proteases within lysosomal and endosomal vesicles (Diment & Stahl, 1985; Guagliardi *et al.*, 1990; Rodriguez & Diment, 1992; Van Noort *et al.*, 1991). In addition, qualitative evidence supports a pivotal role for

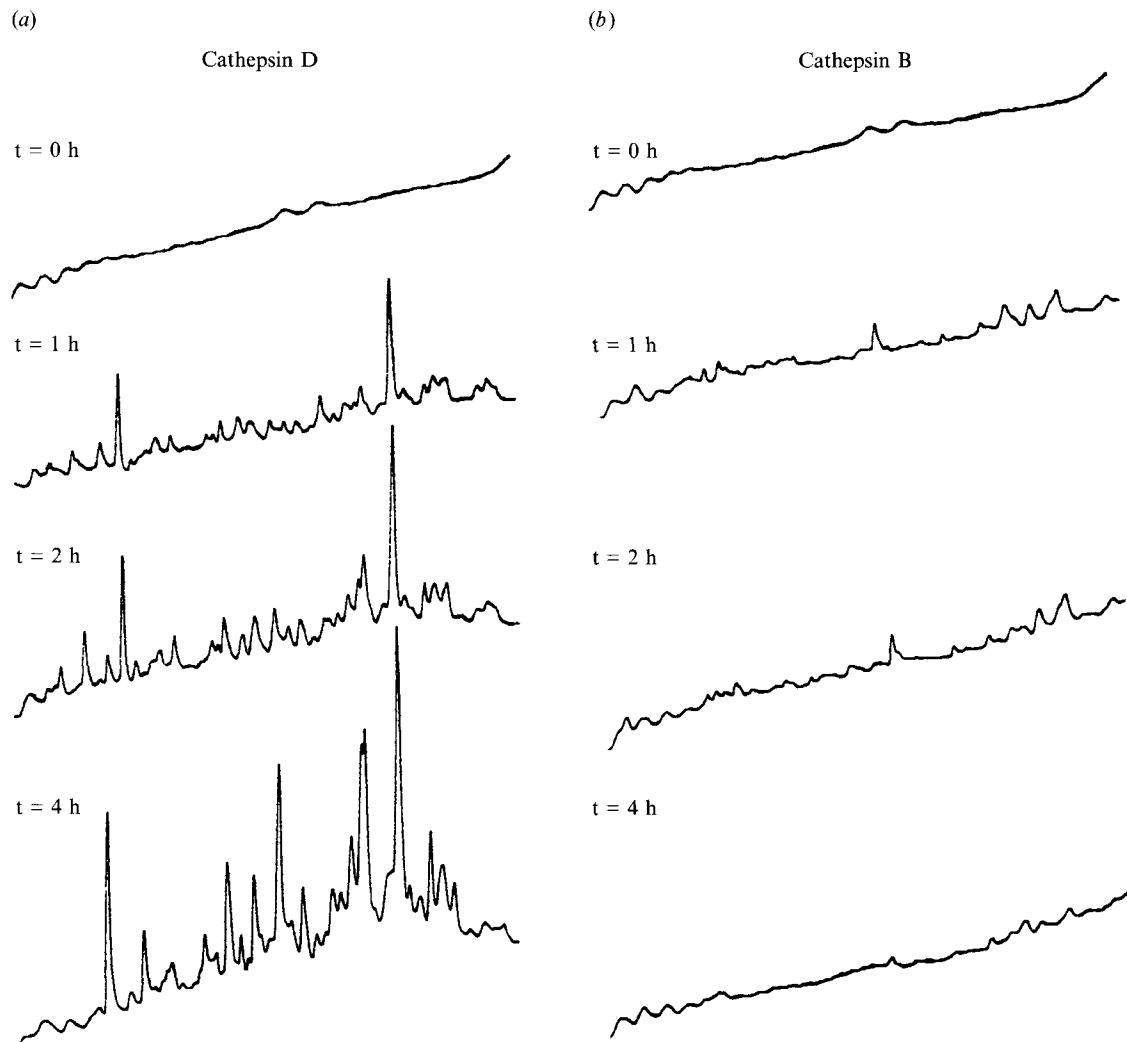


Fig. 1. Reversed-phase HPLC profiles of protein fragments released from purified FMDV by incubation with cathepsin D (a) or cathepsin B (b) for 0, 1, 2 and 4 h.

cathepsin D in processing since the enzyme's selectivity appears to influence the selection of T cell epitopes (Van Noort & Van der Drift, 1989; Van Noort *et al.*, 1991). For the effective release of T cell-stimulatory peptides, various antigens need not only to be subjected to proteolysis, but also to disulphide bond reduction, which may only be effected within late endosomes or lysosome-like vesicles (Peters *et al.*, 1991; Collins *et al.*, 1991). This may well explain why antigens that require reduction, such as bacteria, can only be processed late in the endocytic pathway (Harding *et al.*, 1991; Harding & Geuze 1992), whereas antigens lacking disulphide bonds are processed in early endosomes (McCoy *et al.*, 1993). It should be noted, however, that such different requirements do not necessarily imply the involvement of different proteases in the degradation event.

Our studies aim at the identification of T cell epitopes

of foot-and-mouth disease virus (FMDV) that are relevant in cattle (Van Lierop *et al.*, 1992). Therefore, we examined the applicability of mild *in vitro* treatment of purified FMDV with either cathepsin D or B (with or without a preceding reduction of the virus) for the production and identification of bovine T cell-stimulatory fragments. In addition, we compared the responses to the T cell sites identified in this way with the responses to T cell sites not included within the predominant cathepsin D-released fragments, with respect to their contribution to the total T cell response to inactivated intact FMDV.

Methods

Virus. FMDV strain A₁₀Holland (sequence reference: Thomas *et al.*, 1988) was grown on a baby hamster kidney cell line (BHK-21). The collected supernatant was treated with 0.04 M binary ethyleneimine to

inactivate the virus. All protein material was precipitated twice with 9% and 6% (w/v) polyethyleneglycol 6000, respectively, and adsorbed onto filter-aid as described by Bahnmann *et al.* (1975). The proteins were eluted from the filter-aid for 1 h with elution buffer (50 mM- Na_2PO_4 pH 7.5, 0.2 M-NaCl), cleared by centrifugation for 10 min and precipitated by addition of polyethyleneglycol 6000 up to 9% (w/v). The precipitate was resuspended in elution buffer and intact virus was purified on a CsCl equilibrium gradient in the same buffer (25000 r.p.m., 18 h at 4 °C, Beckman SW28 rotor). The optical density peak (OD_{260}) of intact virus at a density of 1.42 g/ml was pooled. Purity was further confirmed by SDS-urea-PAGE as described by Meloen & Briaire (1980). The preparations were dialysed against elution buffer, precipitated with 2 volumes of acetone at -20 °C and stored at -20 °C until use or resuspended in phosphate buffer (pH 7.5) at a concentration of 1 mg/ml for use in proliferation assays.

Animals. All cattle used in this study were female Dutch Friesian cows belonging to the University of Utrecht herd. The animals were from 5 to 7 years old and they had all been vaccinated annually with the generally used trivalent FMDV vaccine, which is a mixture of strains A₁₀Holland, O₁BFS1860 and C₁Detmold. All animals were last vaccinated 13 months before the start of the experiments. MHC class II typing of these animals was performed as described by Joosten *et al.* (1989) and expressed as DRBF types according to the Fifth International BoLA workshop (Davies *et al.*, 1994). The DRBF types of the animals were 5/7 (animal no. 99), 3/7 (animal no. 95), 3/5 (animal no. 83), 3/7 (animal no. 82), 2/7 (animal no. 87), 2/9 (animal no. 13), 2/8 (animal no. 44) and 4/7 (animal no. 97). According to the MHC class II background the group of animals was found to form a random selection.

Peptides. Peptides were prepared by automated simultaneous multiple peptide synthesis (SMPS). The SMPS set-up was developed using a standard autosampler (Gilson 221) as described previously (Van der Zee *et al.*, 1992). Briefly, for the concurrent synthesis of peptides, standard Fmoc chemistry with Pfp-activated amino acids (Dhbt for serine and threonine) in a six-fold molar excess and Hobt as catalyst were employed. Peptides were obtained as C-terminal amides from 6 mg resin/peptide (0.33 meq./g PAL resin, Millipore). The length of the peptides was between 10 and 20 amino acids. Within each region of interest peptide sequences overlapped for ten or more amino acids. Thus, every possible 11-mer sequence of each identified cathepsin D-released fragment and every 13-mer of the VP4 sequence was contained within a peptide.

The following peptides were synthesized: from VP1, from the amino acid sequence [1-37]: TTATGESADPVTTVENYGGGETQVQRRH-HTDVGFMID, [1-15], [5-19], [10-24], [15-26], [19-33], [20-34], [21-35], [22-36], [23-37]. From sequence [35-65]: IMDRFVKINS-LSPTHVIDLMHTHKHGIVGAL, [35-39], [35-53], [37-51], [39-53], [40-53], [47-60], [49-62], [52-65]. From sequence [77-115]: EIVVR-HDGNLTWVPNGAPEAALSNTSNPTAYNKAPFTRL, [77-88], [81-95], [87-100], [91-105], [95-109], [106-115]. From sequence [113-130]: TRLALPYTAPHRVLATVY, [113-127], [116-125], [116-127], [116-130]. From sequence [120-163]: TAPHRVLATVYN-GTSKYASGSRRGDLGSLATRVATQLPASFN, [120-134], [121-135], [124-138], [127-141], [128-142], [128-157], [130-143], [131-146], [137-146], [137-157], [140-160], [143-157], [143-158], [144-158], [145-159], [147-157], [147-163], [149-163]. From sequence [157-172]: LPASFNYGAIKAQAIH, [157-170], [158-172]. From sequence [194-212]: VSSQDRYKQKIIAPAKQLL, [194-212].

From VP2 sequence [62-69]: FKKFLFDW, [62-69], from sequence [66-98]: LFDWTTDKPFGHLTKLELPTDHHGVFGHLVDSY, [66-80], [69-78], [71-85], [72-86], [74-88], [75-89], [76-85], [76-90], [78-91], [84-98]. From sequence [125-132]: MVPEWKEF, [125-132]. From sequence [198-218]: KVYANIPTYVHVAGELPSKE,

[198-212], [201-215], [205-218], [208-218]. The sequence [54-72]: RVVQAERFFKKFLFDWTTD, 80% identical to an identified T cell epitope in FMDV strain O₁K (Collen, 1991), was also synthesized.

From VP3 sequence [71-96]: TRLLAKFDVSLAAKHMSNTYLS-GIAQ, [71-84], [77-90], [78-92], [79-93], [80-94], [81-95], [82-96]. From sequence [201-221]: VVSASAGKDFELRLPIDPRPQ, [201-216], [206-221], [213-221].

From VP4, from its complete sequence [1-69]: NTGSIINNYYM-QQYQNSMSTQLGDNTISGGSGNEGSTDTTSTHTTNTQNDW-FSKLASSAFTGLFGALLA, [1-15], [4-18], [7-21] and so on to [55-69]. Each peptide synthesized was checked by HPLC.

Reduction and proteolytic digestion of FMDV. Purified FMDV particles, stored in acetone at -20 °C, were pelleted by centrifugation at 3000 g for 30 min and resuspended in PBS at a final concentration of 10 mg/ml. Subsequently, the virus suspension was dialysed against 0.05% (v/v) acetic acid in water. For reduction of viral particles, the FMDV suspension in PBS was supplemented with urea to a final concentration of 8 M and 2-mercaptoethanol to a final concentration of 500 mM, and incubated for 2 h at 37 °C. Extensive dialysis of reduced FMDV particles against three 5000 ml changes of 0.05% acetic acid was performed under a nitrogen atmosphere in order to prevent re-oxidation.

Dialysed FMDV particles were supplemented with sodium acetate/acetic acid buffer pH 5.0 to a final concentration of 80 mM together with either purified bovine cathepsin D (Sigma) or purified bovine cathepsin B (Sigma) to a final concentration of 0.65 units/ml. Proteolysis was allowed to proceed for various periods of time at 37 °C and terminated by the addition of Tris-HCl pH 9.0 to a final concentration of 300 mM and acetonitrile to a final concentration of 50% (v/v). The resulting solution was left at room temperature for 18 h to allow the formation of a precipitate of undigested protein and virus particles which would otherwise interfere with HPLC analysis. The precipitate was removed by centrifugation at 3500 g for 30 min and the resulting supernatant was aspirated with nitrogen in order to remove the acetonitrile from solution.

HPLC analysis of proteolytic digests. For the analysis of the digests as well as for purification of individual FMDV fragments, each digest from which undigested protein and virus particles were removed as described above, was loaded onto a reversed-phase HPLC column (Suprapak PepS, C18, 5 µm, 4.6 × 250 mm, Pharmacia LKB) and eluted with a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid, pH 2.1. The eluate was monitored at 214 nm, collected and lyophilized. This material, dissolved in RPMI-1640 (Gibco), was used in proliferation assays. Individual fragments were purified by additional HPLC runs using gradients of acetonitrile in 50 mM-ammonium acetate pH 5.8. Identification of fragments was based on amino acid composition analysis, performed as described previously (Van Noort *et al.*, 1991). For a number of fragments, the first 10 N-terminal residues were also subjected to amino acid sequencing. Sequencing was performed on an Applied Biosystems model 470A protein sequencer, on-line equipped with a model 120A PTH analyser.

Proliferation assays. Bovine peripheral blood mononuclear cells (PBMC) were isolated from a buffy coat preparation from heparinized blood on a Ficoll-Isopaque gradient (1.078 g/ml; Pharmacia) followed by three washes in RPMI-1640 (Gibco). These PBMC were restimulated *in vitro* by incubating 2.5 × 10⁶ cells/ml culture medium in 96-well round-bottom tissue culture plates (Becton Dickinson) together with 0.25 µg/ml FMDV strain A₁₀Holland in a humidified atmosphere at 37 °C and 5% CO₂. The culture medium consisted of RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (Gibco), 50 IU/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), 2 mM-L-glutamine (Gibco), non-essential amino acids (Flow Laboratories) and 5 × 10⁻⁵ M-

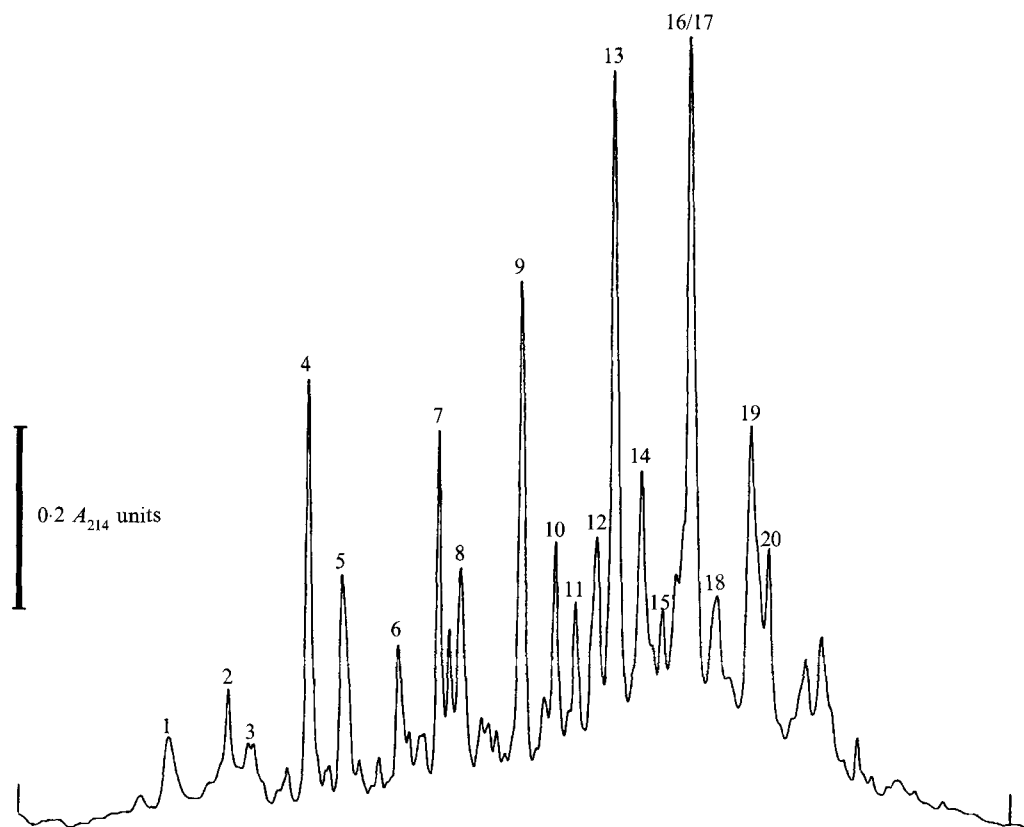


Fig. 2. Reversed-phase HPLC profile of protein fragments released from purified FMDV by incubation with cathepsin D for 4 h. Peak numbers correspond to Table 1.

2-mercaptoethanol (Fluka AG). After 7 days in culture the cells were collected and T cell blasts were separated on a Ficoll-Isopaque gradient (1.078 g/ml; Pharmacia). These cells were cultured for another 7 day at a concentration of 5×10^5 cells/ml in 24-well tissue culture plates (Costar) in culture medium supplemented with 10 IU/ml recombinant human interleukin 2 (IL-2) (Sanofi). Hereafter, cells were tested in a 5 day proliferation assay at a concentration of 2.5×10^5 cells/ml. As APC, freshly isolated irradiated (30 Gy) autologous PBMC (10^6 /ml) were used. Cells were incubated in 96-well flat-bottom tissue culture plates (Costar) at 37 °C and 5% CO₂ in a humidified atmosphere in the presence of different concentrations of purified virus or peptides, different solutions of the viral digests (prepared as described above) or 2.5 µg/ml concanavalin A (Sigma) in a total volume of 200 µl. All cultures were performed in triplicate. During the last 18 h of culture 0.4 µCi [³H]thymidine (sp. act. 1.0 Ci/mmol; Amersham) was added to each well. Cells were harvested using an automatic cell culture harvester (Skatron). Incorporation of label was assessed by liquid scintillation counting in a Betaplate-counter (Pharmacia). Results obtained were presented as the arithmetic mean or as stimulation index (S.I.; the ratio of antigen specific proliferation and background proliferation).

Results

Analysis of proteolytic digests of (reduced) FMDV

Purified FMDV particles were digested *in vitro* with

cathepsin D or cathepsin B for 1, 2 or 4 h. The reversed-phase HPLC profiles of the different digests are shown in Fig. 1. After incubation of the virus for 1 h with cathepsin D, fragments were released causing two predominant peaks in the HPLC profile. Upon prolonged digestion a significant number of other fragments appeared, as shown by the distinct peaks in the HPLC profile after 4 h of proteolysis. In contrast, incubating the virus with cathepsin B did not result in the appearance of any fragment, even after 4 h of incubation. This failure of cathepsin B to cleave FMDV was not due to an inactive state of the enzyme, since a control protein (human haemoglobin) was efficiently cleaved by the same batch of cathepsin B (data not shown).

In order to examine the possibility that reduction of the disulphide bonds within the virus particles would change the efficiency or specificity of the digestions, a chemical reduction of the virus was performed prior to incubation with either cathepsin D or cathepsin B. However, HPLC analysis of the resulting products after the enzymatic digestions revealed profiles that were essentially unchanged compared to the HPLC profiles of the digests of non-reduced virus (data not shown).

Table 1. *Predominant protein fragments released from FMDV after a 4 h mild cathepsin D treatment*

Peak number*	Residues
1	VP3: 215–221
2	VP2: 208–218
3	VP1: 69–72
4	VP1: 194–212
5	VP1: 35–39
	VP1: 118–126
6	VP2: 62–65
7	VP1: 131–157
8	VP1: 54–65
9	VP1: 1–28
10	VP2: 62–68
	VP3: 201–...†
11	VP3: 78–...†
12	VP2: 201–218†
13	VP1: 40–53†
	VP1: 116–126†
	VP2: 125–132
14	VP1: 15–36†
	VP1: 127–157†
	VP2: 69–78
15	VP1: 1–34
16/17	VP1: 1–36
	VP1: 77–107
	VP1: 147–163
	VP1: 158–...†
18	VP1: 77–115
19	VP2: 69–98†
20	VP1: 137–163

*The numbers correspond to the peaks indicated in the HPLC profile shown in Fig. 2.

†Fragments also subjected to amino acid sequencing of the first 10 N-terminal residues. For fragments VP3 [201–...], VP3 [78–...] and VP1 [158–...] the exact C-termini could not be established with certainty.

Analysis of FMDV fragments released by cathepsin D

In order to identify predominant products of cathepsin D-catalysed cleavage, FMDV was mildly digested by cathepsin D for 4 h. Subsequently, the fragments released were freed from residual undigested material by passage over a reversed-phase HPLC column. From a second run, the HPLC profile of which is shown in Fig. 2, predominant fragments corresponding to the 20 peaks indicated in the HPLC profile, were isolated, further purified and analysed for their amino acid composition. In addition, selected fragments were subjected to partial amino acid sequencing. Analyses of three independent digests, of which the HPLC profiles corresponded, resulted in the identification of the 28 fragments listed in Table 1. As can be seen in this table (and in Fig. 4), of the four coat proteins of FMDV, VP1 was the most susceptible to cathepsin D cleavage, VP2 and VP3 were cleaved at a limited number of sites resulting in a few fragments, whereas no fragments from VP4 were recovered.

Proliferative responses to proteolytic digests of (reduced) FMDV

Next, we examined the ability of the cathepsin D-released set of FMDV fragments to trigger FMDV-specific bovine T cells. For this purpose, different dilutions of one of the catheptic FMDV digests, freed from residual undigested material by passage over a reversed-phase HPLC column, were tested in a proliferation assay. In the assay, equal dilutions of a catheptic digest of reduced FMDV and inactivated intact virus were tested. PBMC from eight MHC class II-typed cattle were used as the source of T cells. Prior to the assay these cells were once restimulated with inactivated intact virus *in vitro*. The results of these tests are shown in Fig. 3. For none of the eight animals tested could a significant difference be observed between the proliferative response to the catheptic digest of virus that was pretreated under reducing conditions and the proliferative response to the same amount of digested non-reduced virus. However, the proliferative response to the digest when related to the response to the inactivated intact virus showed distinct individual differences. Four animals were good responders to the digest (Fig. 3*a* to *d*), three could be classified as intermediate responders (Fig. 3*e* to *g*) and one animal appeared to be a low responder (Fig. 3*h*). These differences suggested that not all major T cell sites were equally represented in the catheptic digest.

Proliferative responses to synthetic peptides

Individual cathepsin D-released fragments of FMDV could not be obtained in amounts that would allow screening of their respective antigenicity. Therefore, overlapping sets of synthetic peptides covering their sequences were prepared and tested. To examine the possibility that low responses to the catheptic digest were associated with responses to epitopes not represented in the digest, additional sequences were also synthesized and tested. These included the complete VP4 sequence and the sequence [54–72] of VP2, of which the homologous region in FMDV strain O₁K is known to be recognized by FMDV O₁K-specific bovine T cells (Collen, 1991). In this way the complete sequence of VP4, 95% of VP1, 44% of VP2 and 25% of VP3 was covered. All peptides were tested at three concentrations (5×10^{-5} M, 5×10^{-6} M and 5×10^{-7} M) in a similar proliferation assay to that described above. For this assay, we used PBMC from animal 83, belonging to the group of animals that responded well to the catheptic digest (Fig. 3*c*) and animal 97, being a low responder to the digest (Fig. 3*h*). Clearly positive responses were found to some of the peptides that contained sequences derived from the four regions: VP1 [35–65], VP1

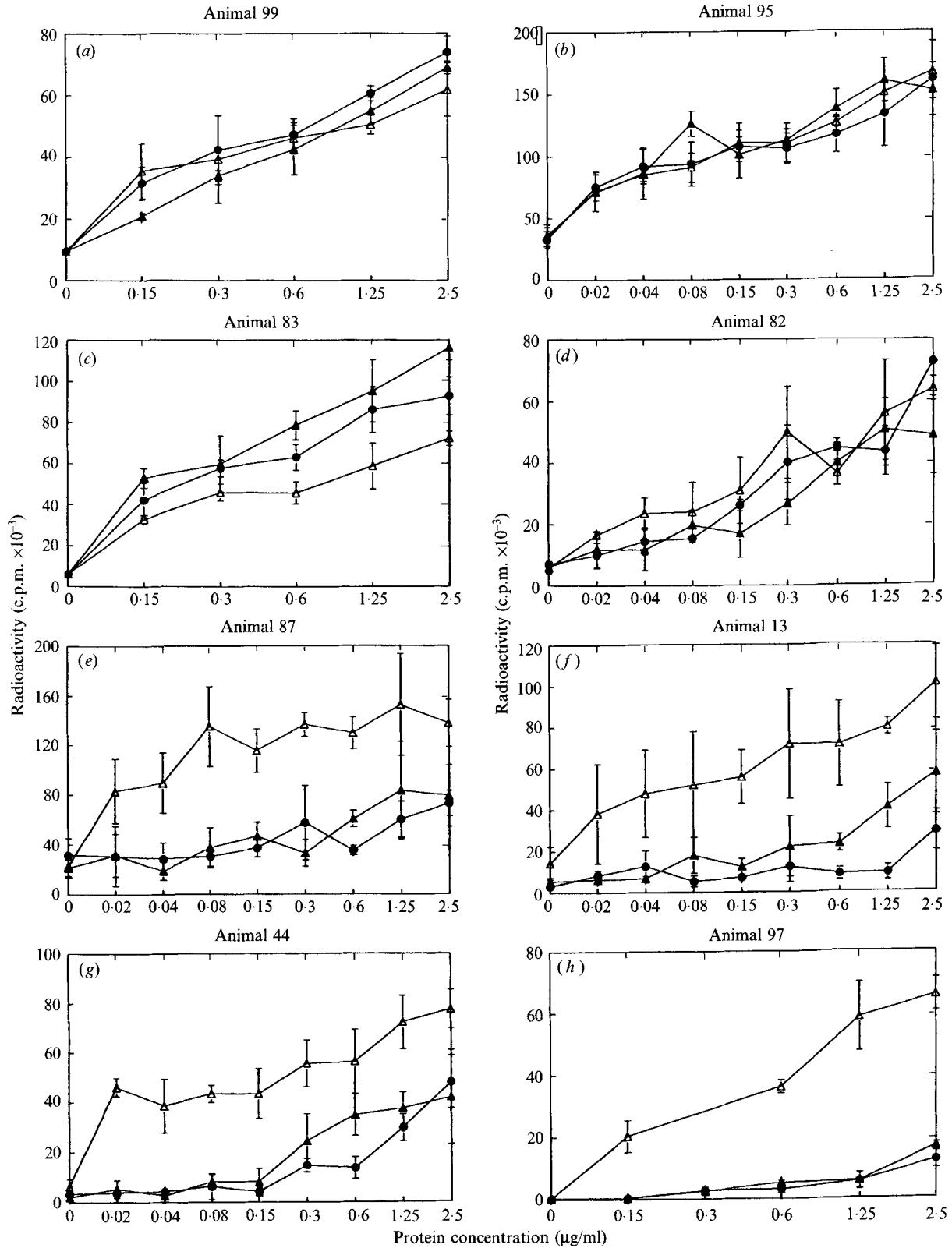


Fig. 3. Proliferative responses of PBMC from eight vaccinated cattle restimulated with FMDV *in vitro*, to whole FMDV (Δ), FMDV digested with cathepsin D (\blacktriangle) and FMDV reduced and digested with cathepsin D (\bullet).

Table 2. Proliferative responses of PBMC from two cattle, 83 and 97, to synthetic FMDV peptides

Peptides*	PBMC S.I.†		Corresponding cathepsin D-released fragments‡
	Animal 83	Animal 97	
VP1 [40-53]	-	4.0	VP1 [40-53]
VP1 [47-60]	7.5	-	VP1 [40-65]
VP1 [87-100]	15	-	VP1 [77-107]
VP2 [74-88]	-	23	VP2 [69-98]
VP3 [78-92]	286	-	VP3 [78-...]
VP2 [54-72]	26	-	None
VP4 [4-18]	80	34	None
Intact FMDV	450	30	

* From the set of overlapping peptides, the peptide inducing the highest proliferative response is presented.

† S.I. values presented are calculated from the means of triplicate cultures at the optimum peptide concentration of 5×10^{-5} M or optimum virus concentration of 2.5 µg/ml. In this test background proliferation (mean of 24 cultures) was 175 c.p.m. for animal 83 and 4830 c.p.m. for animal 97. Standard deviation of the mean was 20% or less in all cases.

‡ Identified cathepsin D-released fragments (see Table 1) based on which overlapping peptides were synthesized.

[77-115], VP2 [66-98] and VP3 [71-96]. Furthermore, the peptide representing VP2 [54-72] and the first three N-terminal overlapping peptides from VP4 were recognized by one or both animals. Table 2 summarizes the results, showing only one peptide per set of overlapping peptides, which induced the highest response at an optimal concentration. At a 10-fold lower concentration of these peptides responses could still be detected (S.I.s > 3). These results gave an indication as to which sites the T cell responses were mainly directed. The most dominant site for animal 83, VP3[78-92], coincided exactly with one of the identified cathepsin D-released fragments, which is also reflected by the high response to the catheptic digest (Fig. 3c). The most dominant stimulatory site for animal 97 was found within the N-terminal part of VP4, which could not be recovered after cathepsin D treatment of the virus. This result is also in agreement with the data presented in Fig. 3(h), showing a low response by this animal to the catheptic digest. In a separate experiment the same set of peptides was tested on lymphocytes from two additional animals (animals 87 and 99). Animal 87 showed responses to peptides corresponding to VP1[35-65] (peptides [40-53] and [47-60] in particular), VP2[66-98], VP2[54-72] and VP4[1-18] (data not shown). From these peptides, VP2[74-88] and VP4[4-18] were most dominantly recognized. This again is in agreement with an intermediate response towards the whole set of cathepsin D-released fragments shown in Fig. 3(e). Animal 99 responded to three regions: VP1[35-65], VP2[66-98] and the N-terminal region of VP4 (data not shown). The highest responses were found to peptides VP1[47-60] and VP2[74-88], which explains the high response of this

animal towards the complete set of cathepsin D-released fragments (see Fig. 3a).

Discussion

The aim of our study was to investigate the usefulness of cathepsin D-released fragments of a complex antigen like FMDV for the identification of dominant T cell sites.

First we had to investigate whether FMDV could be digested *in vitro* by cathepsin D or cathepsin B, proteases that are presumed to fulfil this role during *in vivo* processing (Van Noort *et al.*, 1991). Antigen processing, resulting in immunologically relevant fragments, probably takes place in endocytic vesicles under mild conditions (pH 5 to 6) (Van Noort *et al.*, 1991; Davidson *et al.*, 1990; Marsh *et al.*, 1992). Therefore, *in vitro* digestions were performed at a pH of 5.0. Incubation of FMDV with cathepsin B did not result in the release of any fragment. Lack of any endoproteolytic activity by cathepsin B under these conditions extends previous observations with myoglobin (Van Noort *et al.*, 1991), fructose-1,6-biphosphate aldolase (Bond & Barrett, 1980), lysozyme (Van Noort *et al.*, unpublished results) and influenza virus nucleoprotein (Van Noort *et al.*, unpublished). In contrast, treatment of FMDV with cathepsin D led to the appearance of several fragments that gradually accumulated during the course of mild proteolysis. Although this set of peptides may well be similar to the FMDV fragments generated upon *in vivo* processing, differences may also be expected. First of all, although the method of virus inactivation does not affect the three-dimensional structure of the virus particle or change the structure of the separate VPs, a possible difference in the route of inactivated virus after cell entry might cause a difference in processing. However, since the inactivated virus vaccine has been proven to elicit a protective immune response, T cell sites are presented that induce memory T cells cross-reactive with infectious virus. Others have shown that endogenously synthesized antigen gives rise to a set of MHC class II binding epitopes very similar to those originating from the same antigen administered exogenously (Adorini *et al.*, 1993). Secondly, peptide bonds may be protected from enzymatic cleavage *in vivo* by binding to MHC class II molecules (Mouritsen *et al.*, 1992) or molecular chaperones (Denagel & Pierce, 1992). Thirdly, chemical factors like reducing agents may alter antigen conformation and, therefore, local susceptibility to proteolysis. (Collins *et al.*, 1991). We studied the effect of reduction on the enzymatic cleavage of FMDV. The residues that can form disulphide bonds within the virus particles of FMDV strain A₁₀Holland are Cys-185 in VP1, Cys-120 in VP2 and five Cys residues in VP3. However, reduction of FMDV coat proteins did not

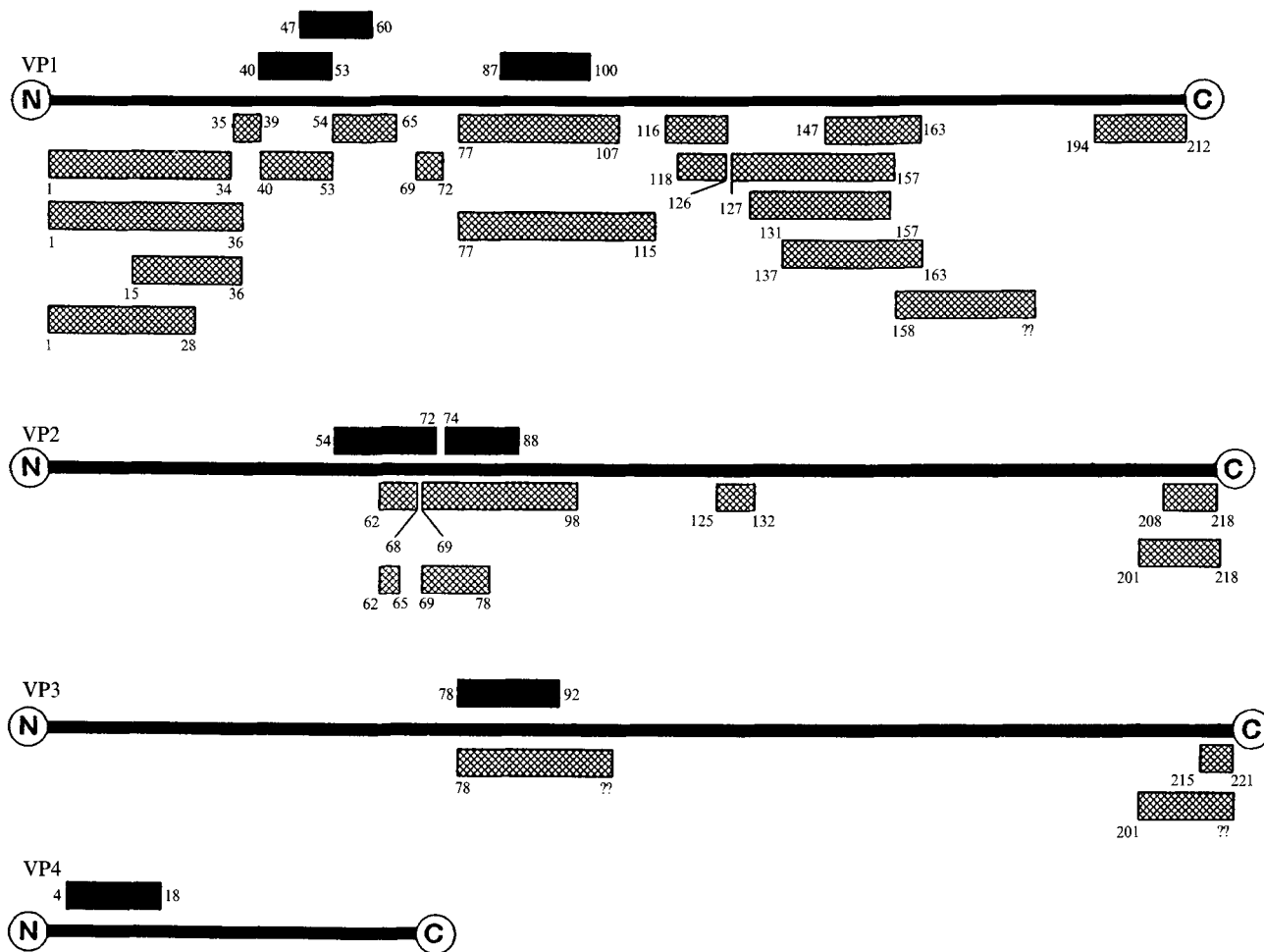


Fig. 4. Released fragments of the four coat proteins of FMDV strain A₁₀Holland identified after a 4 h incubation with cathepsin D (shaded bars) and the T cell sites that have been identified using synthetic peptides (black bars).

result in an increased susceptibility to cathepsin B, nor did it result in a significantly different set of fragments released by cathepsin D as shown by the HPLC profile.

From the catheptic digest of non-reduced FMDV 28 major fragments were identified (delineated in Fig. 4). Most of these were derived from VP1; from the region 127–163 five overlapping fragments were detected. An obvious explanation is the fact that part of this region protrudes from the surface of the virus capsid as a loop, which makes it a region highly susceptible to proteolytic cleavage similar to the C-terminal region of VP1 (Acharya *et al.*, 1989; Strohmaier *et al.*, 1982). Analysis of the two HPLC fractions containing the predominant products of a short 1 h digestion are consistent with the above. The major product within the first HPLC fraction (peak 4) was identified as VP1[194–212]; major products present in the second HPLC fraction (peak 16/17) were identified as VP1[147–163] and a fragment starting from residue 158 of VP1. It is noteworthy that several investigators have identified T cell epitopes on VP1 in the

regions located between residues 150 and 160 and between residues 200 and 212 (Pfaff *et al.*, 1982; Francis *et al.*, 1987; Glass *et al.*, 1991; Van Lierop *et al.*, 1992). However, in these studies, responses towards these two T cell sites were only found after peptide immunization and not after vaccination with inactivated intact virus. Also in our study, peptides representing the cathepsin D-released fragments from these regions were not recognized by PBMC from vaccinated cattle. So, although these sites are readily released from inactivated intact virus by cathepsin D *in vitro*, they do not represent dominant T cell epitopes after vaccination and stimulation with inactivated intact virus. Possibly, these regions are also highly sensitive to proteolysis by other proteases or alternatively, they may be easily bound and thus sequestered by membrane receptors or cytosolic chaperone proteins when presented in the context of complete virus or protein.

To examine whether the cathepsin D-released fragments of reduced and non-reduced FMDV contained

major T-cell epitopes recognized by vaccinated cattle, the complete set of fragments produced as well as separate overlapping synthetic peptides were tested for their ability to stimulate FMDV-specific bovine T cells. The data given in Fig. 3 clearly reveal the presence of immunogenic peptides in the complete cathepsin D-released set of fragments for all eight animals tested. A quantitative comparison with the intact virus as T cell-stimulatory antigen is difficult, since such a comparison of T cell responses is only possible on basis of antigen molarities. In Fig. 3 the total mass of protein within the digests and the intact virus preparation are shown along the horizontal axes. However, a qualitative comparison between individual animals can still be made. Responses to the digests, when related to the responses to the inactivated intact virus, clearly differed between the eight animals. These individual differences were reflected in the response profiles towards synthetic peptides covering the sequences of the 28 fragments analysed plus a set of extra peptides, covering the complete VP4 protein and one already defined T cell site of FMDV strain O₁K, VP2[54–72]. One of the animals (83), shown to be a high responder to the catheptic digest, appeared to predominantly recognize VP3[78–92], corresponding to a cathepsin D-released fragment. For another animal (97), a low responder to the catheptic digest, the virus response appeared to be mainly directed to a T cell site present in VP4, not found within cathepsin D-released fragments. So, whether the most dominantly recognized T cell site was recovered from the catheptic digest or not, appeared to be related to the high or low response towards the digest. The strength of this relationship was further supported by the results obtained with two additional animals. The differences in the response profiles towards the synthetic peptides between the animals is likely to be a consequence of differences in the expressed MHC class II types. This aspect will need further study.

From two out of five T cell sites recovered from cathepsin D-released fragments the N-termini were formed by cathepsin D cleavages, which implicates the direct relevance of cathepsin D in the generation of these T cell sites. T cell epitopes not represented in the digest obtained with cathepsin D alone, may be released by other proteases acting either separately or in concert with cathepsin D during antigen processing. Also for the three T cell sites present in the middle of fragments released by cathepsin D, a further N- and C-terminal degradation by other proteases within the APC may take place.

In conclusion, we have shown that FMDV strain A₁₀Holland can be digested under mild conditions *in vitro* by cathepsin D but not by cathepsin B, with or without preceding reduction of the virus particles. The unseparated set of fragments released by cathepsin D was able to activate FMDV-specific bovine T cells in all

animals tested. Furthermore, five of the major cathepsin D-released fragments contained T cell sites. However, two major T cell sites could not be recovered from the digest. In line with this, the virus-specific response of animals showing a low response to the digest appeared to be shifted towards one or both of these T cell sites. Thus, cathepsin D digestion of an antigen and identification of the fragments released may reveal some immunodominant T cell epitopes but it will fail to identify others.

Note added in proof. The N terminus of VP4 of FMDV may be 16 residues ahead from residue number 1 used in this paper, according to the defined N terminus of VP4 of poliovirus [Chow *et al.* (1987). *Nature* **327**, 482–486].

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