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MHC class II restricted recognition of FMDV peptides by bovine T cells

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SUMMARY

A putative synthetic vaccine for foot-and-mouth disease (FMDV15) has proved less successful in a host species, cattle, than predicted by results in a small-animal model. Possible reasons for this include non-recognition by T cells influenced by major histocompatibility complex (MHC)-linked immune response gene control. It is now possible to type for human leucocyte antigen (HLA) DR-like bovine MHC (BoLA) class II polymorphisms with a one-dimensional isoelectric focusing (IEF) technique. Using this method 14 unrelated cattle were selected with eight different BoLA class II IEF types. After immunization with FMDV15, 13 cattle generated a T-cell response to FMDV15. However, the fine specificity and magnitude of the response was related to BoLA class II type. The non-response by one animal and low response by two other animals were associated with two of the BoLA class II types. Response to the region 149–158 was immunodominant and animals which did not respond to this region had low responses to the whole peptide. Using FMDV-specific T-cell lines five BoLA class II types associated with responder animals were able to present FMDV15 in an MHC class II-restricted fashion, indicating that this peptide is capable of binding to different MHC class II molecules and may account for the broad response observed. The restriction patterns of the lines indicated that the IEF method does not distinguish all functional polymorphisms. At least two of the IEF-defined types could each be split into two distinct specificities and revealed that the three sets of animals with identical IEF types in fact expressed distinct restriction elements.

INTRODUCTION

Synthetic peptide vaccines have been proposed as alternatives to conventional vaccines because of the limitations of the latter. However, the antigenic simplicity of synthetic peptides leads to possible constraints on the design of effective synthetic vaccines, including the requirement for linked T- and B-cell epitopes as well as recognition of T-cell epitopes by all major histocompatibility complex (MHC) haplotypes. However, although MHC-linked immune response (Ir) gene effects have been demonstrated in an inbred mouse model with simple antigens, the importance of MHC polymorphism in the recognition of antigen, in a relevant outbred species such as cattle, is unclear.

Neutralizing antibody and protection are associated with two distinct regions of the VP1 capsid protein of the 01 Kaufbeuren strain of foot-and-mouth disease virus (FMDV).[1,2] A putative vaccine antigen (FMDV15) which comprises these two regions, 141–158 and 200–213, linked with Pro-Pro-Ser and having Cys-Cys and Pro-Cys-Gly at the N-terminus and C-terminus, respectively, in the absence of a carrier, has been shown to induce a protective response in cattle.[2] However, not all animals were protected even though high serum neutralizing titres (SNT) were generated. Also, a high dose of this peptide was required despite being administered in Freund's complete adjuvant. In contrast, complete protection is readily achieved in guinea-pigs with low doses of this peptide or higher concentrations of 141–160 alone.[2] A high proportion of antibodies induced by either of the peptides reacts with the virus in neutralizing and non-neutralizing assays,[3,4] indicating the effectiveness of the peptides as B-cell antigens. Thus, the poorer response of cattle may relate to T-cell recognition influenced by MHC class II-linked Ir gene products. Indeed, Ir gene regulation of the 141–160 antibody response has been reported in mice.[5]

An obstacle to more relevant studies in cattle has been the inability to distinguish polymorphic differences in bovine MHC (BoLA) class II molecules. This has now been resolved by the use of an IEF method, at least as far as products from a DR-like locus.[6] We have shown that these products function as restriction elements.[7] In this paper we have investigated bovine T-cell recognition of FMDV15 and related peptides and demonstrate MHC class II restriction in a natural host for FMDV.

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MATERIALS AND METHODS

Peptides

Peptides were prepared by solid-phase synthesis[8] and correspond to VP1 sequences of FMDV strain 01K (Type 0, subtype 1, strain Kaufbeuren[9]). Peptides which combine discontinuous regions of the protein (e.g. FMDV15) were prepared in a single synthesis. The peptides used in this study are shown in Fig. 1. FMDV15 represents the peptide used by DiMarchi *et al.*[2] and consists of the 200–213 sequence and the 140–158 sequence of VP1 coupled by a Pro-Pro-Ser spacer together with a dicysteine at the N-terminus and Pro-Cys-Gly at the C-terminus. FMDV19 comprises the 141–158 sequence together with Pro-Cys-Gly at the C-terminus. FMDV1.4, 1.3 and 1.2 span regions of the 141–158 sequence and comprise 149–160, 149–163 and 152–163, respectively. FMDV26 comprises the 200–213 sequence only. Finally, FMDV5 comprises the 141–158 sequence coupled to a different sequence of VP1, 21–40.

Source of IL-2

Human recombinant interleukin-2 (hrIL-2) was purchased from Boehringer (Lewes, Sussex, U.K.).

Animals

Friesian (*Bos taurus*) castrated male or female cattle from the research station's herd were used for this study. All the animals were clinically normal and over 6 months of age. For immunization 14 castrated male cattle between 9 months and 12 months were chosen.

Immunization

The animals were immunized subcutaneously with 1 mg FMDV15 (first immunization, Week 0), 0·2 mg FMDV15 (second immunization, Week 13) and 1 mg FMDV5 (third immunization, Week 25) in 1:1 ratio of saline and non-ulcerative Freund's incomplete adjuvant (NUFA), courtesy of Mr Brian D. Morris, Guildhay antisera, University of Surrey, U.K.

Preparation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected just before immunization and at varying times thereafter. PBMC were separated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) as previously described[10] and were resuspended in RPMI-1640 supplemented with 25 mM HEPES, 2 mM glutamine, 10% foetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol and 50 μg/ml gentamycin (Gibco, Paisley, Renfrewshire, U.K.) (complete medium).

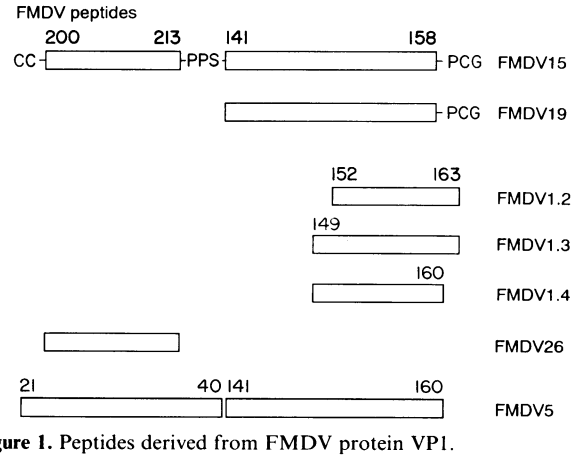

Figure 1. Peptides derived from FMDV protein VP1.

Generation of FMDV15-specific BoCD4$^+$ cell lines

The generation and growth requirements of these lines is similar to those for ovalbumin, as described elsewhere,[11] and are briefly as follows. PBMC from animals immunized with FMDV15, which responded in antigen-dependent proliferation assays,[12] were selected for the generation of BoCD4$^+$ lines, following the method of Kimoto & Fathman.[13] After 7 days culture in the presence of FMDV15 (1 μg/ml), a blast-enriched population was obtained from the proliferating PBMC by separation on a discontinuous Percoll gradient.[14] The T-cell blasts were then alternatively cultured in antigen and irradiated PBMC (5000 rads) or hrIL-2 at weekly intervals. After the third *in vitro* antigen restimulation, the lines were tested or frozen. Resuscitated lines gave similar results to those tested immediately. The phenotype of these cell lines was similar to those described previously.[11] They were >90% BoCD4$^+$ as assessed by the monoclonal antibody (mAb) IL-A11 (this mAb was a kind gift from Dr A. J. Teale, ILRAD, Nairobi, Kenya, and detects the bovine CD4 equivalent).[15] They required autologous MHC class II-positive antigen-presenting cells (APC) for antigen-specific proliferation. No proliferation to an unrelated antigen (ovalbumin) was observed. Background counts were usually less than 200 c.p.m. and the optimal dose of FMDV15 was 1 μg/ml, which was the concentration employed in the experiments reported here.

Proliferation assays

These were essentially as described elsewhere.[11,12] Briefly, PBMC (2×10^5/well), or T-cell lines (1×10^4/well) together with irradiated PBMC (6×10^4/well) (5000 rads), were incubated with antigen, and cell proliferation measured after 5 days or 3 days, respectively, by a final 6-hr pulse with [^{3}H]thymidine (Amersham International, Amersham, Bucks, U.K.) and uptake assessed by liquid scintillation counting.

BoLA class I typing

A micro-lymphocytotoxicity test, as described by Spooner *et al.*,[16] was used to detect all of the internationally agreed workshop specificities.[17] Other sera detecting specificities not yet agreed internationally were given an Ed prefix. All specificities behaved as alleles of a single class I locus.

One-dimensional isoelectric focusing (IEF)-BoLA class II typing

IEF and immunoprecipitation of BoLA class II antigens were carried out according to the method of Joosten *et al.*[6] using a rabbit anti-human HLA-DR antiserum (this antiserum was a kind gift from Dr H. Ploegh, The Netherlands Cancer Institute, The Netherlands) which precipitates BoLA class II molecules. So far, we can detect 13 distinct banding patterns for the β-chain with two bands per haplotype; α-chains appear to be mainly non-polymorphic. The patterns are designated EDF1–EDF13 (E for Edinburgh, D in analogy to the human HLA class II system, and F for focusing).[7]

Statistics

Results were compared using the Student's *t*-test.

RESULTS

The peptides used in this study are shown in Fig. 1 and were tested in the range 0·01–10 μg/ml. Proliferative responses by PBMC were analysed repeatedly from Week 1 to Week 55 after priming (followed by secondary and tertiary immunizations). It should be noted that prior to immunization none of the animals showed any response to FMDV15.

Recognition of FMDV15

Although the response to FMDV15 varied between animals, FMDV15 was immunogenic in 13 out of the 14 animals (Table 1). Peak proliferative responses were observed between 5 and 10 weeks after the first and second immunization (Fig. 2). Responses by T cells from animals 1, 2, 4 and 5 remained high throughout the second immunization period, whereas animals 3, 6, 7, 10 and 11 had sharp peaks which declined rapidly. After the third immunization responses were maintained by animals 1–9 up to the end of the experiment, whereas animals 10–13 showed lower responses which declined to low levels by Week 55. Nonetheless, animals 10–13 did show significant responses at a higher dose of FMDV15 (10 μg/ml) (Table 1). Animal 14, on the other hand, did not respond even after the third immunization and even with the highest dose of FMDV15 employed (10 μg/ml).

The responding cells were mainly BoCD4$^+$ T cells, as assessed by FACS analysis (results not shown). Also, responses to FMDV15 by nylon wool-purified T cells (containing $\geqslant 90\%$ T cells and $\leqslant 1\%$ macrophages or B cells)[10] together with autologous accessory cells exactly parallelled those with untreated PBMC (results not shown).

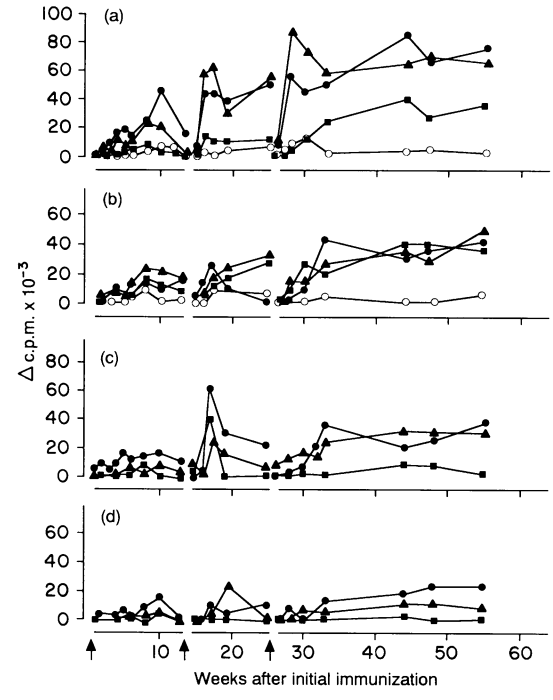

Figure 2. Time-course of proliferative responses by T cells from all 14 animals to FMDV15 (1 μg/ml) after each immunization. The arrows indicate the time-points for each immunization. (a) Responses by T cells from animals 1 (●), 2 (▲), 9 (■) and 12 (○). (b) Responses by T cells from animals 3 (●), 4 (▲), 5 (■) and 13 (○). (c) Responses by T cells from animals 6 (●), 7 (▲) and 10 (■). (d) Responses by T cells from animals 8 (●), 11 (▲) and 14 (■).

Table 1. Peak response to FMDV15, 19 and 1.2, 1.3, and 1.4 after the 3rd immunization

			FMDV peptide* Δc.p.m. × 10^{-3}				
Donor	MHC class II	MHC class I	15	19	1.2	1.3	1.4
1	3, 7	w11, w20	87	80	79	81	91
2	2, 6	w10, Ed99	108	30	12	12	13
3	7, 8	w11, w14	67	61	2	8	26
4	4, 6	w10, w15	67	42	0	0	−4
5	4, 6	w15, w18	41	40	2	5	4
6	2, 7	w13, Ed99	38	21	7	0	8
7	6, 8	w10, w11	42	13	7	8	7
8	4, 7	w10, w14	35	38	1	3	3
9	3, 7	w11, w32	45	26	1	9	14
10	6, 8	w10, –	19	5	0	0	0
11	2, 5	w32, Ed99	34	14	1	0	−1
12	5, 6	w10, w32	34	27	−1	−2	1
13	8, 11	w14, w18	4	10	0	0	0
14	5, 11	w18, –	2	1	0	0	0

* Peptide conc.^{n} was 10 μg/ml.

Δc.p.m. = c.p.m. of test − c.p.m. with medium alone.

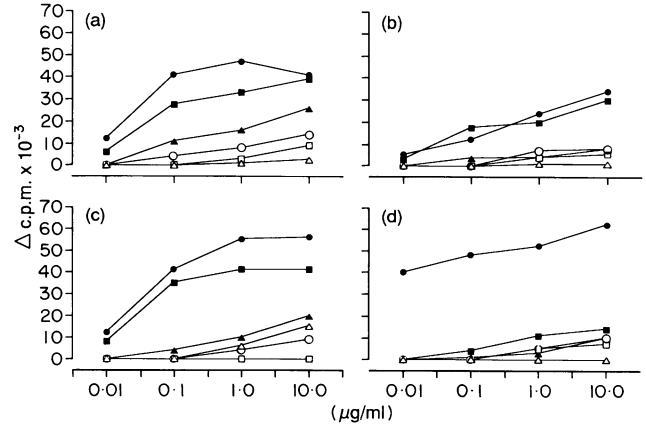

Figure 3. Proliferative responses by T cells from animals 1 (EDF3, 7), 2 (EDF2, 6), 3 (EDF7, 8), and 9 (EDF3, 7) (a, d, c, and b, respectively) to FMDV peptides at Week 33. (●) FMDV15; (■) FMDV19; (□) FMDV1.2; (○) FMDV1.3; (▲) FMDV1.4; (Δ) FMDV26. Δc.p.m., c.p.m. in the presence of antigen − c.p.m. with medium alone. SD were $\leqslant 10\%$.

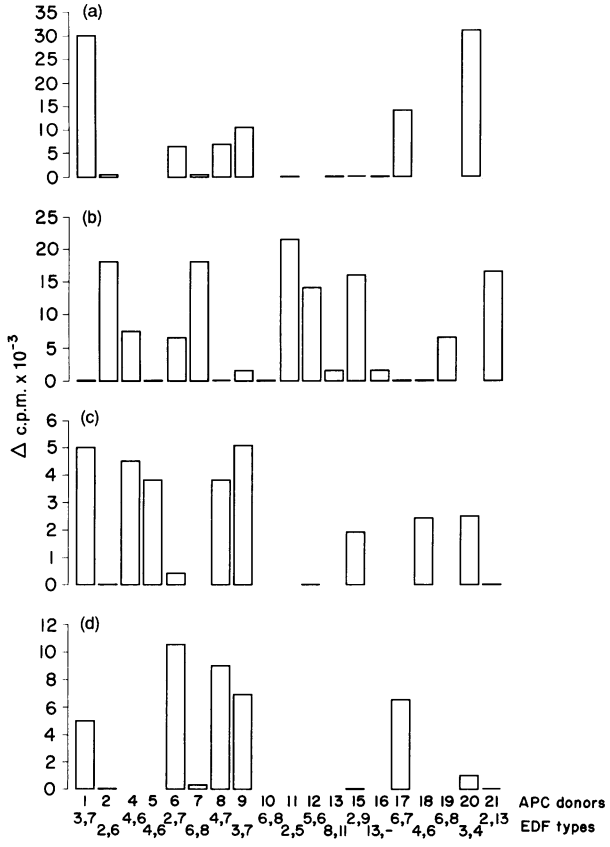

Figure 4. Proliferative responses by FMDV15-specific T-cell lines derived from animals 1 (EDF3, 7), 2 (EDF2, 6), 8 (EDF4, 7), and 9 (EDF3, 7) (a, b, c and d, respectively) to FMDV15 (1 μg/ml) with a panel of APC. EDF (BoLA class II) types of the panel of APC donors are shown.

Immune responses to regions of FMDV15

All 13 of the animals responding to FMDV15 also responded to the 141–158 sequence (FMDV19) (Table 1). Figure 3 shows typical dose–responses by four responder animals. All four animals responded significantly to FMDV15 down to 0·01 μg/ml ($P \leqslant 0{\cdot}01$). Proliferation to FMDV19 was essentially similar to FMDV15 by PBMC from animals 1, 3, and 9 (Fig. 3a, c, b, respectively), whereas a markedly lower, though still significant, response to FMDV19 was observed with animal 2 (Fig. 3d) ($P \leqslant 0{\cdot}05$ at 0·1 μg/ml). Of the 14 animals tested only one animal, 3, responded consistently to a peptide comprised of the 200–213 sequence alone (FMDV26) (Fig. 3c) ($P \leqslant 0{\cdot}02$ at 1 μg/ml).

To analyse the T-cell recognition of the 141–158 region more fully, three smaller peptides containing this sequence were tested—FMDV1.2 (152–163), FMDV1.3 (149–163) and FMDV1.4 (149–160). Of the 13 responder animals, eight (animals 1–3 and 5–9) responded to one or all of these peptides (Table 1) ($P \leqslant 0{\cdot}05$). Six animals (4, 10–14) did not respond to the three shorter peptides, FMDV1.2, 1.3 or 1.4 (Table 1). With the exception of animal 4, this group had low or non-responses to FMDV15 (Fig. 2). The magnitude of response to the smaller peptides was lower than that observed to FMDV15 (Table 1 and Fig. 3). Even at the highest dose employed, 10 μg/ml, the response to these three peptides was usually less than that seen at 0·1 μg/ml of FMDV15 (Fig. 3).

Bovine MHC (BoLA) class II influence on bovine T-cell recognition of FMDV15

Responsiveness. All EDF5- and/or EDF11-positive animals had low responses to FMDV15 and did not respond to the shorter peptides. Animal 14 possessed both of these haplotypes and was the only animal which did not respond at all to FMDV15.

All EDF3- and/or EDF7-expressing animals responded to at least one of the three shorter peptides. However, the remaining EDF types tested, i.e. EDF2, 4, 6 and 8, were found in both the responder and non-responder groups for the shorter peptides. Since the animals were heterozygous, it was not possible to determine directly if all of the EDF haplotypes in the responder group were capable of presenting FMDV15. Furthermore, three sets of animals (1 and 9; 4 and 5; 7 and 10) had identical EDF haplotypes but responded to the peptides differently, suggesting that not all restriction elements are distinguished by the IEF method. To address these issues, T-cell lines were established from some of the responder animals. To date no FMDV15-specific T-cell lines have been established from the low-responder group (animals 10–14).

MHC class II restriction. The T-cell lines were ⩾90% BoCD4$^+$ and required accessory cells together with antigen for proliferation (results not shown). Their restriction patterns were analysed using a panel of defined APC (Fig. 4). None of the lines responded to any of the panel of APC in the absence of antigen. All positive reactions with each cell line were highly significantly different from their controls ($P \leqslant 0{\cdot}001$). Only APC expressing EDF types in common with the individual cell lines were able to present FMDV15 with one exception—the T-cell line derived from animal 8 (EDF4, 7) proliferated to FMDV15 with APC 15 (EDF2, 9) (Fig. 4c). However, not all APC which shared EDF types with the cell lines were able to present FMDV15. Thus lines 1 and 9 (both EDF3, 7) reacted to all APC expressing EDF7 (Fig. 4a, d) but only line 1 reacted to APC 20 (EDF3, 4). Cell line 2 (EDF2, 6) reacted strongly with several EDF6-expressing APC, i.e. APC 5, 7, 12 and 19, but was not stimulated by APC 4, 10 or 18 (Fig. 4b).

DISCUSSION

This report investigates bovine T-cell recognition of a putative vaccinal peptide, FMDV15, in relation to MHC class II type. Friesian cattle were used and thus were of the same breed as the animals used in the original study on FMDV15.[2] Moreover, they were unrelated and were selected to minimize as many background effects as possible, e.g. they were all male and of a similar age. The composition of BoLA class II types in the group selected (Table 1) reflected the most frequent haplotypes in our herd (R. A. Oliver, unpublished observations). Animals were immunized with 1 mg uncoupled FMDV15 in FIA. This dose is of the order previously shown to induce a strong immune response in cattle.[2] FMDV15 contains T-cell epitope(s) which are recognized by T cells from 13 out of 14 cattle. The cattle were heterozygous for EDF type and thus expressed at least two potential restriction elements for presentation of FMDV15. Furthermore, the broad response to this peptide suggests that it is capable of binding to several different bovine MHC class II molecules. With most animals, FMDV19 elicited essentially similar responses to FMDV15, indicating that the region 140–

158 contained major T-cell determinants for cattle. This region is a focus for T cells from other species, including laboratory mice and guinea-pigs.[18] It contains structural features associated with T-cell epitopes, including amphipathic α-helices and a Rothbard predicted epitope[19] 154–157 (KVAR).[20] Smaller peptides containing this sequence were recognized by eight animals. The magnitude of response was considerably lower with these peptides except with animal 1, suggesting that the removal of C-terminal Pro-Cys-Gly influenced the response. These shorter peptides also did not contain the conserved Arg-Gly-Asp (RGD) sequence, which is involved in virus attachment to cells[21] and may therefore enhance binding of the larger peptides, FMDV15 and FMDV19, to APC. The remaining five animals (4, 10–13) which did not respond to the shorter peptides all recognized FMDV19 and must therefore be recognizing a T-cell epitope which requires residues within the sequence 141–148. Other than animal 4, these animals also had relatively low T-cell responses to FMDV15 (and correspondingly low responses to FMDV19). This suggests that the region containing the Rothbard predicted epitope is immunodominant. As suggested by Kojima *et al.*[22] for whole proteins, recognition of this region would determine the magnitude of response to the whole peptide, FMDV15.

Although all three shorter peptides contained the Rothbard predicted epitope, the sequences to either side of the epitope appeared to influence recognition (Table 1). Of particular interest, the addition of three residues to the C-terminus, i.e. 160–163 (FMDV1.3) reduced responsiveness by animals 3 and 9, and loss of residues 149–151 (FMDV1.2) almost totally ablated T-cell receptor (TcR) recognition. The presence or absence of these residues may hinder MHC class II binding or TcR recognition, possibly through their influence on peptide conformation,[23] underlining the stringent structural requirements for effective peptide vaccines. The 200–213 sequence coupled to a carrier induced very low levels of neutralizing antibody in guinea-pigs,[1] whereas as a free peptide it failed to induce anti-viral or anti-peptide antibody and to protect.[24] However, when co-linearly synthesized to 141–158 it improved the efficacy of the 141–158 peptide.[2] The 200–213 sequence contains no structures currently associated with T-cell epitopes,[25] although a response was seen consistently with animal 3. The 21–40 sequence, on the other hand, does contain an epitope(s) recognized by bovine T cells.[25] In order to determine whether the 200–213 sequence played any role in the T-cell recognition of 141–158, the third immunization was with FMDV5, which consisted of the 141–160 sequence co-linearly synthesized with 21–40. The majority of animals (1–9) maintained T-cell responses to FMDV15 and FMDV19 up to Week 55 (and indeed to Week 110, results not shown). Thus memory T cells had been generated. However, animals 10 and 11 had low responses which were not sustained, although they had responded to FMDV15 and to the 141–158 sequence after the second immunization. This suggests that, for these animals, the 21–40 sequence had adversely affected the recognition of 141–158. This may be because the 21–40 sequence alters the conformation of the peptide, thereby affecting the binding of 141–158 to MHC molecules, or the exact epitope generated by processing may be different. Thus the context in which an epitope is seen may affect its recognition, as has been described for other antigens,[23,26] and has considerable implications for the design of effective vaccines. In terms of MHC class II-linked Ir gene effects, EDF5 and 11 may confer low responsiveness. Animal 14 was the only animal which did not respond at all and possessed both of these types. Of the low responder animals, 10–13, three of these animals possessed EDF5 or EDF11. The poor immunogenicity of FMDV15 in these animals may be related to the ability of these MHC alleles to bind this peptide, since determinant selection appears to correlate with T-cell responsiveness.[27] Alternatively, FMDV15 may induce an MHC-linked Ts response. However, the low responsiveness cannot simply be attributed to dominant suppression, since these animals responded to FMDV15, albeit at a relatively low level.

Apart from EDF3 and EDF7, which were found exclusively in the group which responded to the shorter peptides, FMDV1.2–1.4, the other EDF types were associated with both the responder and non-responder groups.

T-cell lines were generated from some of the animals in the responder group to further investigate MHC class II-linked control of T-cell recognition of FMDV15. The response by these T-cell lines confirmed that EDF2, 3, 4, 6 and 7 can present FMDV15. Thus several different bovine class II molecules can bind this peptide. This is in contrast to most other peptide antigens, although 'promiscuous' binding to MHC class II molecules has been reported for a malaria peptide[28] and two tetanus toxoid peptides.[29] Nonetheless the cattle group contained low responders, and variation in response to the shorter peptides FMDV1.2–1.4 was observed. These results imply that the interactions between peptide and different MHC class II molecules are different. Even so, peptide binding to multiple MHC class II molecules is promising for the development of synthetic vaccines for outbred species.

Although the cell lines, with one exception, only responded to FMDV15 in association with APC expressing shared IEF types, they did not respond to every APC with common EDF types. In particular, only cell line 1 and not cell line 9 (both EDF3, 7) responded to APC 20, which apparently shared EDF3 with both cell lines. Furthermore, marked differences in the ability of APC from the other two sets of animals with apparently identical EDF types to present FMDV15 to cell line 2 (through EDF6) were observed. Thus APC from animals 4 (EDF4, 6) and 7 (EDF6, 8) induced a positive response whereas, in contrast APC 5 (EDF4, 6) and 10 (EDF6, 8) were unable to present to cell line 2. The inability of the APC 5 to present FMDV15 to line 2 was not due to an intrinsic defect in presentation, since APC 5 was able to present FMDV15 to cell line 8 (via EDF4). Taken together these results suggest that the IEF method does not distinguish all MHC class II haplotypes. Either different restriction elements have identical pI points or FMDV15 has been presented through non-DR-like restriction elements. Whichever is the case, it provides an explanation for the differences in T-cell response seen in Fig. 2 and Table 1 by the three sets of animals, which have apparently identical EDF haplotypes (i.e. animals 1 and 9; 4 and 5; 7 and 10). This is in contrast to the presentation of another antigen, ovalbumin, where there appears to be closer association between the EDF types and T-cell response.[7] Only one α/β product per haplotype is detected with the IEF technique,[6] whereas evidence for more than one BoLA class II locus at the genetic level has been reported.[30–32] Furthermore, patterns of reactivity with BoLA class II-specific alloclones also indicate that more than one restriction element is expressed per haplotype.[33] This is the subject of further research.

In conclusion, FMDV15 does contain bovine T-cell epitopes and the MHC class II type of the animal profoundly affects its response to this peptide, both in terms of fine specificity and in magnitude of response. Nonetheless this peptide does bind to several different BoLA class II molecules. Current research is investigating the relationship of the T-cell response to FMDV15 to the production of serum neutralizing antibody and protection. These results have far-reaching implications for the design of a more effective vaccine against FMDV in cattle, and indeed for the design of subunit vaccines for any outbred species.

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