INDUCTION OF EFFECTIVE CROSS-REACTIVE IMMUNITY BY FMDV PEPTIDES IS CRITICALLY DEPENDENT UPON SPECIFIC MHC-PEPTIDE-T CELL-INTERACTIONS

Citation for published version:

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Published In:
Immunology

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Induction of effective cross-reactive immunity by FMDV peptides is critically dependent upon specific MHC–peptide–T cell interactions

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SUMMARY

BoCD4⁺ T-cell clones specific for a peptide derived from foot-and-mouth disease virus envelope protein, VP1 (FMDV15) were generated from two responder cattle. One animal was a high and the other was an intermediate responder in terms of both T-cell and antibody responses. However, both animals had identical major histocompatibility complex (MHC) class II DR-like types (DRBF3,6) according to a one-dimensional isoelectric focusing method which distinguishes DR-like alleles. In contrast, mixed lymphocyte reaction (MLR) responses indicated that they shared only one haplotype (DRBF3) and anti-DRBF6 alloclones also differentiated between the animals. This suggested that the animals differed at a non-DR-like locus. Restriction patterns of FMDV-specific clones derived from these animals indicated that FMDV15 was presented by the non-DR-like class II molecules associated with DRBF6. Only one clone, derived from the high responder animal, was restricted to DRBF3. Thus products from the non-DR-like locus (probably DQ-like) are functionally important for presentation of FMDV peptides. Furthermore, the allelic differences generated by the alloclones are also critical for peptide binding. The majority of clones from the high responder animal recognized an immunodominant region containing a Rothbard epitope whereas none of the clones from the intermediate responder did so. This suggests that the region recognized by T cells, which is dependent upon MHC type, influences the B-cell response and thus the degree of protection obtained. This has major implications for rational vaccine design involving T- and B-cell epitopes.

INTRODUCTION

The interaction between major histocompatibility complex (MHC) class II molecules, antigenic peptides and clonotypic T-cell receptors (TcR) are essential to most if not all immune responses. The role of different MHC class II loci and alleles in Ir gene regulation of T-cell recognition is well established. In contrast, how this control affects the outcome of an immune response in terms of generating protective immunity, is poorly understood. We are investigating how variations in MHC–T cell interactions lead to differences in effective immunity using a model system—bovine T-cell responses to a peptide derived from foot-and-mouth disease virus (FMDV15)—which also has direct practical implications.

Neutralizing antibody is believed to be the most important effector mechanism against FMDV infection. The major B-cell site has been located to the loop region of virus envelope protein (VP)1 and is present within the synthetic peptide, FMDV15. Although this peptide was designed as a potential synthetic vaccine, it has proved less successful in the host species than predicted by results in a small animal model. The relative importance of T-cell recognition and function in protection against the virus may be more important than previously realized. We have shown that the synthetic peptide contains bovine T-cell epitope(s). In addition, other structural proteins of the virus contain T-cell epitopes.

We have shown that the recognition of FMDV15 by bovine T cells is under bovine MHC (BoLA) class II-restricted control. Polymorphism of the expressed products of a bovine DR-like locus alters both the magnitude of the T-cell response and the region recognized. These locus products are detected by a one-dimensional isoelectric focusing (IEF) method and we have shown that they function as restriction elements. However, two animals which had the same MHC class II DR type as defined by IEF had different T-cell and antibody responses. Restriction patterns with cell lines derived from these two animals suggested that they may in fact express different MHC class II types. This paper extends these findings and shows that other class II products act as restriction elements important in presenting FMDV15. Furthermore, we suggest that MHC control of the cellular response is important in determining the humoral response. Different T-cell epitopes may be more or less effective in inducing appropriate T-cell help for B cells to make neutralizing antibody.

Received 15 November 1993; accepted 1 February 1994.

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MATERIALS AND METHODS

Peptides
Peptides were prepared by solid-phase synthesis and correspond to VP1 sequences of FMDV strain O1K (type O, subtype 1, strain Kaufbeuren).3 Peptides which contain discontinuous regions of the protein (e.g. FMDV15) were prepared in a single synthesis. FMDV15 represents the peptide used by Di Marchi et al.3 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. FMDV15, 19 and 26 were kindly donated by Dr R. DiMarchi (Eli Lilly, Indianapolis, IN) and FMDV1.2–1.4 were kindly donated by Dr T. Doel (AFRC IAH, Pirbright, U.K.). The region 140–158 contains structural features associated with T-cell epitopes including amphipathic α helices and a Rothbard predicted epitope.1,12

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 castrated male cattle between 9 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 incomplete Freund’s adjuvant courtesy of Mr B. D. Morris (Guildhay antisera, University of Surrey, U.K.).

Preparation of peripheral blood mononuclear cells (PBMC)
PBMC were separated on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) as previously described and were resuspended in RPMI-1640 supplemented with 25 mM HEPES, 2 mM glutamine, 10% fetal calf serum (FCS), 5 × 10⁻⁵ M 2-mercaptoethanol and 50 μg/ml gentamycin (Gibco BRL, Inchinan, U.K.) (complete medium).

Generation of FMDV15-specific BoCD4⁺ clones
BoCD4⁺ clones were generated from PBMC from two selected animals as described in the text which produced both T-cell and antibody responses to FMDV15 following immunization. The initial in vitro expansion was essentially as described previously for FMDV-specific T-cell lines.4 PBMC were first cultured with FMDV15 (1 μg/ml) for 7 days, followed by recombinant human interleukin-2 (rhIL-2) (20 U/ml) for a further 7 days. The blasting cells were then cloned at 0.3 cells/well in 96-well round-bottomed plates together with irradiated autologous PBMC (10⁵/ml), rhIL-2 (20 U/ml) and peptide (1 μg/ml). They were fed at 7-day intervals with irradiated autologous PBMC, rhIL-2 and peptide. After 14–21 days growing clones were split and after a further 7 days were transferred to 24-well plates (Nunc, Gibco BRL). Clones with rapidly growing characteristics were then transferred to 6-well plates. The phenotype of the clones was determined by indirect immunofluorescence and FACS analysis to Glass and Spooner.14 They were ≥ 90% BoCD4⁺, as detected by the monoclonal antibody (mAb) IL-A11;15 BoCD8⁻, as detected by the mAb IL-A5116 and MHC class II⁺ (8–55%), as detected by the mAb J11.17 The clones were MHC class II restricted as determined by blocking with anti-MHC class II and anti-MHC class I mAb as previously described.18

Proliferation assays
These were essentially as described elsewhere.19 Clones were rested prior to use in proliferation assays for 6–8 days after the addition of antigen-presenting cells (APC) and FMDV15. Briefly, T-cell clones (1 × 10⁵/well) together with irradiated PBMC (6 × 10⁶/well) (5000 rads) and rhIL-2 (10 U/ml) were incubated with antigen, and cell proliferation measured after 3 days by a final 6-hr pulse with [³H]thymidine (Amersham International, Amersham, U.K.) and uptake assessed by liquid scintillation counting.

MLR
These were carried out as described previously.20 Responders and stimulators were cultured together for 6 days and proliferation measured as described above.

BoLA class I typing
A micro-lymphocytotoxicity test as described by Spooner et al.21 was used to detect all of the internationally agreed workshop specificities.22

BoLA class II typing by one-dimensional IEF
IEF and immunoprecipitation of BoLA class II antigens were carried out according to the method of Joosten et al.7 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. Distinct banding patterns are seen for the β chain with two bands per haplotype; α chains appear to be mainly non-polymorphic. The patterns are currently designated as DRBF types according to the fifth BoLA workshop.23

BoLA class II typing by BoLA class II-specific alloreactive clones
This was carried out essentially as described by Glass, Millar and Oliver.20 Briefly, bovine BoLA class II-specific clones were generated from responder PBMC, differing from stimulator PBMC by BoLA class II type (as defined by the above IEF method) and not BoLA class I type (as defined by the above micro-lymphocytotoxicity test). For this study, the responder: stimulator pair was class II typed as DRBF7:8, DRBF6:7, and both class I typed as A18 (A6), A31. The phenotypes of the alloreactive T-cell clones were determined as described above and were similar to the FMDV-specific clones. They were MHC class II restricted as described previously.20

Proliferation assay with alloreactive clones
Clones (2 × 10⁴/well) and a panel of BoLA class I and IEF class II typed stimulators (irradiated PBMC) (1-2 × 10⁵/well) were incubated together with 20 U/ml rhIL-2 in quadruplicate cultures in 96-well flat-bottomed plates (Nunc, Gibco
RESULTS

Although animals 10795 and 10814 were BoLA class II typed by IEF as DRBF3,6, their immunological responses to immunization with a peptide derived from FMDV VP1, FMDV15, were markedly different. Peripheral blood T cells from 10795 consistently showed significantly higher responses to FMDV15, FMDV19 and FMDV1.4 compared to those from 10814 (Table 1). Furthermore antibody levels correlated with T-cell responses. SNT from 10795 were higher after the second and third immunization than those from 10814 (Table 1).

The one-dimensional IEF method appears to detect polymorphism in expressed β chains from one locus. However cells from 10795 and 10814 reacted strongly to one another in one-way MLR (Table 2). Neither reacted to stimulator cells (11179) typed by one-dimensional IEF as homozygous for DRBF3 which suggested that they express identical BoLA class II molecules associated with DRBF3. Thus they must express differences in their BoLA class II molecules associated with DRBF6 which can be recognized by T cells.

To investigate these differences further, BoCD4+ alloclones were generated which recognize DRBF6. Two distinct sets of clones were produced. The first set ('A') reacted to the majority of DRBF6-expressing stimulators whereas the second set ('B') had a more restricted recognition pattern (Table 3). Neither set recognized any non-DRBF6-expressing stimulators but did not react to all DRBF6 stimulators. Of particular importance

| Table 1. Comparison of T-cell and antibody responses by 10795 and 10814 |
|-----------------|-----------------|-----------------|-----------------|
| Animal          | Immunization   | FMDV15         | FMDV19         | FMDV1.4         |
| 10795           | 1              | 46             | ND†            | ND              |
|                 | 2              | 58             | 28             | ND              |
|                 | 3              | 145            | 123            | 62              |
| 10814           | 1              | 9              | ND†            | ND              |
|                 | 2              | 13             | 11             | ND              |
|                 | 3              | 23             | 20             | 4               |

* Δ c.p.m., c.p.m. of test – c.p.m. with medium alone.
† ND, not done.

Table 2. MLR responses with 10795 and 10814

<table>
<thead>
<tr>
<th>Stimulators (DRBF class II type; BoLA class I type)</th>
<th>(c.p.m.)</th>
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</thead>
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<tr>
<td>10795 (DRBF3,6; A11,A36(A20))</td>
<td>10814 (DRBF3,6; A11,A32)</td>
</tr>
<tr>
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<td>1136</td>
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<tr>
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<td>47,739</td>
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<td>11179</td>
<td>16,392</td>
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10795 was only positive with the ‘A’ set of clones whereas 10814 was positive with both ‘A’ and ‘B’ sets of clones. A further allo clone ('C') had an even more restricted pattern—it only reacted to three DRBF6-expressing stimulators, including 10814, in the panel used. However it also reacted with a non-DRBF6-expressing stimulator, 10552 (typed by IEF as DRBF4,8).

Next, BoCD4+ FMDV-specific clones were generated from 10795 and 10814. The clones only proliferated to antigen in the presence of appropriate APC; no responses to allo APC in the absence of antigen were seen. Their restriction patterns were analysed using a panel of IEF and alloclone defined APC. All of the clones tested, except one (see below), were restricted to the DRBF6 haplotype (Figs 1 and 2). The sets of clones derived from the two animals had very distinct patterns of response with respect to DRBF6-expressing APC. All clones from 10814 only reacted to FMDV15 in the presence of self-APC or APC which were positive for both ‘A’ and ‘B’ sets of alloclones (Fig. 1).

Although all the clones from 10814 had identical restriction patterns, the DRBF6 haplotype-restricted clones from 10795 fell into three distinct groups. Of the 26 clones which were tested, 25 reacted to FMDV15 in the presence of self-APC or APC which stimulated the ‘A’ set of alloclones (see Fig. 2a–d for the patterns of selected clones). Fifteen clones were more extensively tested with a larger panel of typed APC. Six of these reacted with an additional three DRBF6 expressing APC (Fig. 2a). Of the nine clones which did not react with this group of three APC, two clones reacted with another DRBF6+ APC (5608) (Fig. 2b) and two reacted with 10554 (Fig. 2c). Both APC were negative with all alloclones. Two clones were tested and were positive with 10552 which is DRBF4,8 and DRBF6 but which was positive with alloclone ‘C’ (see above) (see Fig. 2d for one of them). Unfortunately the animal is no longer available to carry out further testing. Two APC which were DRBF6+ but not recognized by any of the alloclones did not act as APC for any of the FMDV-specific clones. Only one clone tested was DRBF3 restricted (Fig. 2e). This clone was the only 10795-derived clone which responded to FMDV15 when presented by 10814 or 11057; both APC must be presenting FMDV15 via MHC class II molecules associated with the DRBF3 haplotype and not the DRBF6 haplotype.

Many of the clones were also tested to see which region(s) of FMDV15 they recognized. Of the 19 clones tested from 10795, only one did not recognize FMDV19 (Table 4). Furthermore, the response to FMDV19 with 17 of these clones closely
paralleled the response to FMDV15 (Fig. 3). Twelve clones recognized FMDV1.2–1.4. One clone (1A-3B7) recognized FMDV26 (Fig. 3b). The restriction pattern for this clone is shown in Fig. 2c. Sixteen clones from 10814 were tested. The majority (10/16) recognized FMDV19 although three clones had a much lower response to this peptide (Table 4 and Fig. 3). Six clones did not recognize FMDV19 or FMDV26. Of particular importance, none of the clones derived from 10814 recognized any of the shorter peptides.

DISCUSSION

This study extends our findings on bovine T-cell recognition of a putative vaccinal peptide, FMDV15 in relation to BoLA class II type. FMDV-specific clones from two animals with apparently identical class II type, as detected by a one-dimensional IEF method, were characterized in relation to their restriction patterns and antigen specificity. Although these two animals had identical DRBF type they produced different T-cell and antibody responses. One animal was a high responder and the other was an intermediate responder. Subsequently, positive MLR responses between these two animals indicated that they could not in fact be identical for all expressed class II molecules (Table 2). Furthermore in a previous study we showed that FMDV-specific lines derived from the two animals described here had slightly different class II restriction patterns. At present the only reliable method for detecting expressed polymorphisms of bovine class II molecules is a one-dimensional IEF technique which we have shown detects functionally important restriction elements. It is assumed that this method detects products from a DR-like locus as the expressed polymorphisms correlate with DR-associated restriction fragment length polymorphism (RFLP) differences. However evidence at both the genomic level and with cDNA indicates that at least another locus is expressed and is polymorphic. Furthermore there appear to be strong associations between DR and DQ haplotypes.

The MLR results with the two FMDV-immunized animals indicated that they were identical for all class II molecules associated with DRBF3. Certainly no splits in DRBF3 have been observed within these animals which, in combination with the data from the IEF studies, suggest that DRBF4 and DRBF6 are the only loci expressed in the two animals.

![Figure 1](image-url)  
**Figure 1.** Proliferative responses by a typical FMDV15-specific clone derived from 10814 to FMDV15 (1 μg/ml) with a panel of APC. DRBF types and alloclass II types of APC donors are shown. ND, not done; (−) negative response. SD ≤ 10%.

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<tr>
<th>Stimulator</th>
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* Positive responses are shown in bold type.
Induction of cross-reactive immunity by FMDV peptides

Figure 2. Proliferative responses by typical FMDV15-specific clones derived from 10795 to FMDV15 (1 µg/ml) with a panel of APC. As Fig. 1, DRBF and alloclass II types of APC donors are shown. (a–e) The variation in restriction patterns seen with different clones. The frequency of clones with these patterns is shown.

been detected by anti-DRBF3 allocloones20 and the latest BoLA workshop reports a single haplotype associated with DRBF3.23 Analysis of the restriction patterns of the FMDV-specific clones revealed that only one was restricted to DRBF3. This is in contrast to T-cell lines generated from these two animals which were restricted to both DRBF3 and DRBF6.4 Possibly the selection procedure for the clones biases T-cell reactivity to certain restriction elements or the frequency of DRBF3-restricted T cells is much lower than that for DRBF6. Limiting dilution analysis of the bulk T-cell population with DRBF6 or DRBF3 homozygous APC may help to answer this question.

The results with both the allocloones and FMDV-specific clones suggest that DRBF6 is not associated with a single haplotype. There are several possibilities to explain these results. DRBF6 has several variants which have identical pi, share allo-epitopes and have distinct allo-epitopes, and have different peptide-binding sites. This is unlikely as most of the amino acid changes that affect binding of peptide also alter the charge.31 Furthermore, although it is possible for two β chains
to have identical pI (e.g. HLA Dw4 and Dw14\textsuperscript{22}), it has never been reported that two variants of an allelic product have identical pI. Alternatively the 'A' type alloclones may see one molecule and the 'B' type alloclones another. Thus 10814 would express both the 'A' molecule and the 'B' molecule. However the FMDV-specific clones derived from 10795 which are restricted to 'A' do not recognize FMDV15 in association with 10814. It seems more likely that the reaction patterns seen with the alloclones are revealing a second locus which codes for products able to present FMDV15. These second locus products are in strong linkage equilibrium with DRBF6. Certainly there are several different RFLP DQ types associated with DRBF6.\textsuperscript{23,25}

Several of the clones derived from 10795 had a different restriction pattern in that they also reacted with additional DRBF6 expressing APC. This suggests that there must be further class II alleles which are associated with DRBF6 and are able to bind and present FMDV15.

Thus the IEF method does not distinguish all functionally important restriction elements. The evidence from both the alloclones and the FMDV-specific clones suggests that other locus products also act as restriction elements. Currently we are developing new methods to detect these functionally important molecules.\textsuperscript{33}

The specificity of the T-cell clones from the two animals was also influenced by the usage of different restriction elements. The majority of clones from 10795 recognized peptides containing the Rothbard predicted epitope. This specificity mirrored the responses found with bulk PBMC.\textsuperscript{4} Some of the clones did not respond to the shorter peptides and must therefore be recognizing T-cell epitope(s) within the sequence 140–149. One clone reacted with FMDV26 which does not contain any features currently associated with T-cell epitopes. In contrast, none of the clones derived from 10814 recognized the shorter peptides and several clones did not even recognize FMDV19. As these clones did not recognize FMDV26, they must be recognizing an epitope which is only present in the 40-mer peptide—the most likely candidate would be around the PPS spacer.

The reactivities of the 10795 clones in relation to their restriction patterns are more complex than those with 10814. This may be because 10795 expresses several restriction elements which can bind and present FMDV15 whereas with 10814 only one restriction element can do so. It might have

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* Frequency refers to the number of clones tested which showed the pattern.
† Positive responses are shown in bold type.

Figure 3. Proliferative responses by different T-cell clones to FMDV peptides. (a and b) were derived from 10795 and (c) from 10814. (●) FMDV15; (◇) FMDV19; (▲) FMDV1.4; (○) FMDV1.3; (●) FMDV1.2; (△) FMDV26. SD ≤ 10%.
been expected that the clones with slightly different patterns might have recognized different regions of FMDV15. More defined peptides may answer this.

If the frequencies of the clonal specificities mirror those in the bulk population this may explain why 10814 has a lower T-cell response than 10795. The region containing the Rothbard predicted epitope appears to be immunodominant and as suggested by Kojima et al., the ability to recognize immunodominant epitopes may determine the magnitude of response to the whole peptide. In addition the antibody responses by 10795 are much higher than those for 10814, suggesting that the region recognized by T cells influences the humoral response. Possibly the type of T cells induced may be different. Although the division into Th1 and Th2 for BoCD4+ T cells has not been reported it is likely that differential cytokine production plays an important role in determining B-cell activity. It has been reported that the generation of Th1 or Th2 responses to a single peptide in mice is dependent upon MHC class II type. Amadori et al. have shown a correlation between protection against FMDV and production of IL-2 and interferon-γ by T cells in vitro. Furthermore through the cytokine network, T cells can determine the isotype and therefore the effector function of antibody. The isotype of antibody produced may be of particular importance in protection against FMDV. Thus there may be a relationship between the induction of protection and the interaction between MHC–peptide–TCR. Currently we are investigating the cytokine profiles of the different sets of clones.

In summary, we have shown that more than one BoLA class II product is expressed per haplotype and more importantly they act as restriction elements for FMDV15. Thus Ir genes are an important consideration for vaccine design as expression of different alleles may determine whether or not an effective immune response is generated.

ACKNOWLEDGMENTS

The authors would like to thank Mr R. A. Oliver for one-dimensional IEF typing and Mrs A. G. Morgan for BoLA class I typing. We are grateful for the gifts of FMDV peptides from Eli Lilly and Dr T. Doel, AFRC IAH. We would also like to thank ILRAD for providing mAb.

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