THE INFLUENCE OF MHC POLYMORPHISM ON THE SELECTION OF T-CELL DETERMINANTS OF FMDV IN CATTLE

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The influence of MHC polymorphism on the selection of T-cell determinants of FMDV in cattle

M.-J. C. VAN LIEROP,* P. R. NILSSON,† J. P. A. WAGENAAR,* J. M. VAN NOORT,‡ J. D. M. CAMPBELL,§ E. J. GLASS,§ I. JOOSTEN¶ & E. J. HENSEN* **Department of Immunology, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, †Department of Animal Husbandry, Agricultural University, Wageningen, ‡TNO Prevention and Health, Leiden, the Netherlands, §AFRC Roslin Institute Edinburgh, Roslin, UK, and ¶Transplantation Serology Lab, University Hospital Nijmegen, the Netherlands*

SUMMARY

There is a quest for the development of a new generation of vaccines consisting of well-defined subunit antigens. For a number of practical reasons it is attractive to develop vaccines on the basis of synthetic peptides. However, their efficacy may be limited by genetic restrictions imposed on T-cell recognition via major histocompatibility complex (MHC) polymorphism, as shown by many studies using inbred animal species. To study the effect of MHC polymorphism in an outbred species, we selected four cattle homozygous for different A-DR-DQ haplotypes, and another four cattle which shared one haplotype in combination with a haplotype of one of the MHC homozygous animals. We analysed responses to synthetic peptides comprising defined T-cell epitopes of foot-and-mouth disease virus (FMDV) in this selected group of FMDV-vaccinated cattle. This analysis shows that even in outbred animals, MHC polymorphism influences the responses to synthetic peptides. Interestingly, one of the peptides, VP4[20–34], was recognized in association with at least four different MHC haplotypes. Fine specificity analysis of this peptide revealed subtle shifts in the core epitope recognized. All peptides that induced lymphocyte proliferation *in vitro* were found to induce a T-helper type-1 (Th1) type of response, irrespective of the MHC haplotype involved. Together, these data support the notion that individuals carrying distinct MHC types can be vaccinated successfully by vaccines that include only a limited number of peptides. In the design of a peptide vaccine against FMDV we suggest inclusion of the highly conserved VP4 sequence 20–34.

INTRODUCTION

One of the novel approaches in vaccine research is the development of vaccines consisting of well-defined subunits of pathogen-derived antigens. Most attractive is the development of vaccines based on synthetic peptides comprising one or more B-cell epitopes and one or more T-cell epitopes of the pathogen. The advantages of such peptide vaccines include reduced costs and improved safety and quality in comparison to vaccines comprising the whole pathogen. They also comply with the growing requirement for marker vaccines that can discriminate between vaccinated and infected individuals. Moreover, by peptide vaccination it is possible to manipulate the immune system in such a manner that the production of protective neutralizing antibodies is induced,[1] or that a non-responder to native antigen is converted into a responder.[2]

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Correspondence: Dr E. J. Hensen, Department of Immunology, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, PO Box 80.165 3508 TD Utrecht, the Netherlands.

However, especially in the development of such peptide vaccines, three aspects need special attention. First of all, polymorphism in pathogen-derived proteins often occurs in those parts of the proteins which are involved in immune recognition. In particular, RNA viruses, including foot-and-mouth disease virus (FMDV), contain regions with extremely high sequence variability.[3] Consequently, T cells primed and antibodies elicited by a vaccine composed of sequences selected from such highly variable regions of the virus might not cross-react with virus occurring in the field.

A second major aspect to consider is the variability of the host immune response. A T-helper cell only recognizes an antigenic peptide in association with one of the major histocompatibility complex (MHC) class II products, expressed by antigen-presenting cells (APC).[4] Each individual expresses its own set of MHC products coded by genes with a large allelic variation (i.e. MHC polymorphism). Hence, the MHC-restricted T-cell response frequently implies an individual-specific T-cell response towards peptides. This has been clearly demonstrated in many studies in which inbred mice strains were used.[5,6]

Finally, the type of T-cell help induced by a peptide vaccine also has to be considered. Different cytokine profiles produced by T-helper cells lead to different effector functions.[7] Again, studies in mice suggest that the type of effector function might also be controlled by MHC class II. In these studies a single peptide was able to elicit different types of T-cell help in different strains of mice.[8]

Here we studied the influence of MHC polymorphism on T-cell responses to peptides after vaccination with the whole pathogen in an outbred species, i.e. cattle. It has already been shown that T-cell responses are under control of MHC class II in cattle.[9–12] Extensive studies on the MHC typing of cattle have revealed a high polymorphism comparable to that in humans.[13] Upon detailed MHC typing of a large cattle herd, vaccinated with whole inactivated FMDV, we were able to select four animals homozygous for A-DR-DQ haplotypes and four related heterozygous animals. In this way proliferative and cytokine responses to three previously defined FMDV T-cell epitopes[14] in association with six different MHC class II (DH) haplotypes could be examined.

MATERIALS AND METHODS

Virus

Viral antigen was prepared as described previously.[14] Briefly, FMDV strain A_{10}Holland was grown on a baby hamster kidney cell line (BHK21). Collected supernatant was treated with 0·04 M binary ethyleneimine to inactivate the virus, and proteins were precipitated twice with 9% and 6% (w/v) polyethyleneglycol (6000 MW), respectively. Intact virus was purified on a $CsCl_2$ equilibrium gradient. Purity was confirmed by SDS–urea–PAGE, as described by Meloen & Briaire.[15] The preparation was dialysed against phosphate buffer (pH 7·5), precipitated with 2 volumes of acetone at −20° and resuspended at a concentration of 1 mg/ml for *in vitro* restimulation of bovine peripheral blood mononuclear cells and proliferation assays.

Peptides

VP1[35–53] (IMDRFVKINSLSPTHVIDL), VP2[74–88] (PFGHLTKLELPTDHH) and VP4[20–34] (SIINNYYMQQYQNSM) were synthesized by standard solid-phase Fmoc chemistry.[16] These peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) and the sequence was checked by amino acid composition analysis. Ten overlapping 15-mers, two 10-mers and one 7-mer covering the VP4 amino acid sequence 17–40 (indicated in Table 5) were prepared by automated simultaneous multiple peptide synthesis, as described previously.[14,17] All peptides were synthesized with an amide group at the C-terminus.

Animals

Eight Holstein–Friesian cows were used. These animals were selected from the Wageningen Agricultural University (WAU) herd, the Netherlands. Selection was based on MHC type and family relationship. Four animals were homozygous for both MHC class I and class II, the other four animals were MHC heterozygous. This latter group included the four MHC class II haplotypes of the homozygous group, plus two additional ones. Three of the heterozygous animals had inherited the same paternal haplotypes as three animals of the homozygous group (Table 1). The age of the animals varied from 3 to 5 years. They all had been vaccinated at least once with the generally used trivalent FMDV vaccine, which is a mixture of strains A_{10}Holland, O_1BFS1860 and C_1Detmold, and were last revaccinated with monovalent vaccine type A, 6 months before the start of the experiments.

Table 1. Interrelationship and MHC class II haplotypes of the selected cattle

Animal no.*	Sire-code father†	Sire-code via dam‡	Paternal haplotype	Maternal haplotype
A1983	026B	021A	DH7A§	DH7A
A2399	060A	046A	DH8A	DH8A
A2346	060B	035B	DH22B	DH22B
A2390	051A	045A	DH24A	DH24A
A2396	060A	18*4	DH8A	DH18A
A2420	060B	011A	DH22B	DH7A
A2351	057B	159A	DH22H	DH18A
A2362	051A	159A	DH24A	DH18A

* Animals A1983, A2399, A2346 and A2390 were homozygous for MHC class I and MHC class II haplotypes. Animals A2396, A2420, A2351 and A2362 were heterozygous. Two extra DH haplotypes (22H and 18A) were included in this latter group.

† Code number of the fathers; note that three fathers of the homozygous animals were shared by three animals from the heterozygous group.

‡ Code number of the sire of which the haplotype was inherited via the mother.

§ Class II haplotype (DH), as shown in Table 2.

MHC typing

Class II polymorphism was established by serological, biochemical and DNA typing techniques. Serological typing was performed by the two-colour fluorescence technique (TCT), as originally described by van Rood *et al.*,[18] with minor modifications as described by Nilsson *et al.*[19] Biochemical typing [immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF)] was performed as described by Joosten *et al.*[20] and Davies *et al.*[21] The MHC class II antigens were precipitated with 3 µl of the monoclonal antibody IL-A21 (a gift of Dr A. J. Teale, ILRAD, Nairobi, Kenya). Molecular typing was performed by two methods: detection of DRB3 polymorphism by polymerase chain reaction–restriction fragment length polymorphism (DRB3-PCR-RFLP) and detection of polymorphism of DQA and DQB by RFLP analysis. DRB3-PCR-RFLP typing was carried out essentially as described by van Eijk *et al.*[22] DQ-RFLP was performed as described by Siguardardottir *et al.*[23] For DQA–DQB typing, first and second domain (exon 2 and exon 3) DQA and DQB probes were used (clone DQA: W1, clone DQB: Y1). The sizes of the molecular weight fragments were adjusted on the basis of the BRL/Lifetechnologies (Baltimore, MD) 1 kb ladder. The nomenclature used for serotyping (Dw, Dc), 1D-IEF (DRBF) and DQA–DQB-RFLP was based on the nomenclature proposed at the Fifth International BoLA Workshop reported by Davies *et al.*[24] The nomenclature used for DRB3-PCR-RFLP was based on the one proposed by van Eijk *et al.*[22] Based on the combination of results of these four typing methods,

Table 2. Defined MHC class II haplotypes (DH)

DH*	Ds (serology†)	DRBF (IEF†)	DRB3 (PCR-RFLP†)	DQA (RFLP†)	DQB (RFLP†)
7A	Dw2	02	7	2	2
8A	Dw1; Dc12	06	8	12	12
18A	Dw1,4; Dc7	04	18	5	5
22B	Dw1; Dc9	07	22	9B	9B
22H‡	Dw1, Dc9	07	22	9B	9A
24A	Dw3	03	24	1A	1

* Class II haplotype (DH) reference numbers, according to the Fifth International Bovine Lymphocyte Antigen (BoLA) workshop.[24]

† The method of typing is shown between parentheses.

‡ This haplotype was not officially defined during the Fifth International BoLA workshop. The nomenclature used is as suggested by this workshop.[24]

class II haplotypes were defined. For each class II haplotype a D-haplotype (DH) code comparable to the one introduced in the class II report of the Fifth International BoLA Workshop[24] was used (Table 2). As MHC class I and class II are strongly linked, additional class I typing of the eight animals used in the study and class I and class II typing of related animals further confirmed the defined MHC class II haplotypes.

Proliferation assay

Proliferation assays were performed as described previously.[14] Briefly, peripheral blood mononuclear cells (PBMC) from the eight FMDV-vaccinated cattle were restimulated by *in vitro* incubation with 0·25 μg/ml viral antigen for 7 days. After an expansion period of another 7 days in medium containing 10 IU/ml recombinant human interleukin-2 (IL-2) (Sanofit, Toulouse, France), cells were tested in a proliferation assay: cells were incubated for 5 days in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA) at a concentration of $2{\cdot}5 \times 10^5$ cells/ml in the presence of freshly isolated irradiated autologous PBMC (1 : 1) and different concentrations of viral antigen or peptides. All cultures were performed in triplicate. During the last 18 hr of culture 0·4 μCi [^{3}H]thymidine (specific activity 1·0 Ci/mmol; Amersham International, Amersham, UK) was added to each well. Incorporation of label was assessed by liquid scintillation counting in a Betaplate-counter (Pharmacia, Uppsala, Sweden). Results were obtained as mean c.p.m.

Cytokine assays

For measurement of interferon-γ (IFN-γ), IL-2 mRNA and IL-4 mRNA, cells were pretreated as described above for the proliferation assay. Incubations with peptides were also performed in 96-well flat-bottomed plates (Costar) at an equal concentration. However, to minimize contamination of the RNA samples with RNA from APC, only adherent cells (from 10^5 irradiated PBMC/well) were used as APC. After 24, 48 and 72 hr of incubation with peptides, non-adherent cells were harvested and washed with cold phosphate-buffered saline (PBS) for RNA isolation, and supernatants were collected for determination of IFN-γ. Different dilutions of the supernatants were assayed in duplicate for IFN-γ using a commercial bovine IFN-γ EIA kit (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) according to the manufacturer's instructions. The use of this kit was described for the first time by Rothel *et al.*[25]

Table 3. Cytokine primer sequences

mRNA	Strand	Sequence
Actin	+	5′ CTGGCACGACACCTTCAACGAG 3′
	–	5′ AGCCAAGTCCAGACGCAGGATG 3′
IL-2	+	5′ AAGTCATTGCTGCTGGATTTAC 3′
	–	5′ CCTGTAGTTCCAAAACGATTCTC 3′
IL-4	+	5′ GCATTGTTAGCGTCTCCTGGTAAAC 3′
	–	5′ CTTCATAATCGTCTTTAGCCTTTCC 3′

\+ reads 5′–3′.
– reads 3′–5′.

Total RNA was isolated from 10^7 cells by the use of RNAzol™ B (Campro Scientific, Veenendaal, the Netherlands) according to the manufacturer's instructions. The amount of RNA was determined by OD_{260} and, if available, 10 μg of each sample was checked by electrophoresis in 2% agarose/20% formaldehyde/20 mM 3-morpholino propane sulphonic acid (MOPS)/5 mM sodium acetate/1 mM EDTA gel. Three samples of 5 μg RNA were used for reverse transcriptase-mediated synthesis of cDNA using the SuperScript™ pre-amplification system (Gibco BRL, Paisley, UK) with oligo d(T) as a primer, according to the manufacturer's instructions. From each reverse transcriptase reaction mixture (volume 20 μl) 5-μl samples were subjected to PCR. Cytokine primers were designed from published cDNA sequences for bovine IL-2[26] and IL-4,[27] using PC/Gene release 6.7 software (IntelliGenetics Inc., Mountain View, CA), and were synthesized by OSWEL DNA services (University of Edinburgh, Edinburgh, UK). Sequences are shown in Table 3. Primers did not amplify products from genomic DNA. Actin primers were included as a control for quality of the cDNA product and PCR. PCR was performed at a final concentration of 10 mM Tris–HCl pH 8·3, 50 mM KCl, 0·1 mg/ml gelatin, 8 mM $MgCl_2$, 0·2 mM dNTP, 0·5 μM of the 5′ and 3′ primer, 2·5 U Thermus aquaticus DNA polymerase (Taq DNA polymerase) (Gibco BRL) in a total volume of 50 μl. The mixture was overlaid with paraffin oil (Sigma, St Louis, MO) and then amplified using a Techne PHC-3 thermal cycler. The amplification consisted of 30 cycles of the following profile: denaturation at 95° for 1 min, primer annealing at 55° for 1 min, and extension at 72° for 1 min. Twenty-five microlitres of each PCR reaction mixture was electrophoresed in 2% agarose gels in Tris borate/EDTA buffer. Gels were stained with ethidium bromide and photographed. Samples were indicated positive for the relevant product when bands of the expected size were visible.

RESULTS

In this study the influence of six bovine MHC class II haplotypes on the proliferation and cytokine production of T cells in response to three T-cell sites of FMDV (VP1[35–53], VP2[74–88] and VP4[20–34]), was examined. The six MHC class II haplotypes were present in eight cattle (Tables 1 and 2). PBMC from these cattle were restimulated once *in vitro* with

Table 4. Proliferation of PBMC from selected cattle to intact inactivated FMDV and to peptides, representing three T-cell sites of FMDV

Animal no.		A1983	A2399	A2346	A2390	A2420	A2396	A2351	A2362
DH*		7A/7A	8A/8A	22B/22B	24A/24A	22B/7A	8A/18A	22H/18A	24A/18A
BG†		4547	660	1285	6438	355	8603	123	4890
FMDV-antigen‡	Conc. 2·5 μg/ml	**42**	**68**	**17**	**21**	**122**	**23**	**57**	**32**
VP1[35–53]§	10^{-5} M	**6·9**	1·2	1·1	0·3	**5·7**	**12**	**86**	**28**
	10^{-6} M	**6·6**	1·1	1·5	0·5	**3·0**	**20**	**62**	**23**
	10^{-7} M	**3·6**	1·6	0·8	0·4	1·0	**20**	**48**	**18**
VP2[74–88]	10^{-5} M	1·8	2·0	**15**	0·5	**303**	1·3	**204**	2·4
	10^{-6} M	1·5	2·3	**8·3**	0·7	**282**	1·0	**198**	1·8
	10^{-7} M	1·3	1·8	**4·7**	0·9	**75**	1·8	**101**	1·7
VP4[20–34]	10^{-5} M	**31**	**66**	**7·1**	0·4	**114**	**8·7**	**151**	**26**
	10^{-6} M	**24**	**44**	**3·0**	1·1	**13**	**5·3**	**74**	**16**
	10^{-7} M	**17**	**11**	2·7	1·0	**4·2**	**3·1**	**27**	**4·3**

* Class II haplotypes of the animals as shown in Table 1 and Table 2.

† BG, background proliferation of PBMC presented as c.p.m. The value obtained is the mean of 12 cultures.

‡ Proliferation to purified FMDV strain A_{10}Holland presented as stimulation index (SI = proliferation to antigen/background proliferation).

§ Proliferation to three molar concentrations of the peptide indicated, also presented as SI.

Bold numbers indicate SI values of 3·0 or higher. The standard deviation of the mean was 20% or less.

purified FMDV. Thereafter, cells were incubated with each peptide, representing one of the three T-cell sites, during which both proliferation and cytokine production were measured. Proliferation assays were performed using cells that were cultured for 5 days in the presence of different concentrations of each peptide or intact inactivated FMDV. Results are shown in Table 4. All cattle responded very well to intact FMDV: in all cases the stimulation index was greater than 15. Seven out of the eight animals also responded to one or more of the peptides. Peptide VP1[35–53] was recognized by PBMC from animals carrying the DH7A or DH18A haplotypes. VP2[74–88] was recognized by PBMC from animals carrying the DH22B or DH22H haplotypes. Notably, peptide VP4[20–34] was recognized by PBMC from most animals, indicating a promiscuous recognition in association with haplotypes DH7A, DH8A, DH22B and DH18A. The animal homozygous for the DH24A haplotype did not recognize any of the peptides.

Apart from a clear selectivity in the responses to the peptides, a difference in the magnitude of the responses was also observed. Animals carrying the DH7A haplotype responded more strongly to the VP4 than to the VP1 peptide, whereas the opposite proved to be the case for two out of three animals carrying the DH18A haplotype. In animals carrying the DH22B or DH22H haplotypes, the response towards the VP2 peptide was most dominant, exceeding the response to the VP4 peptide. Similarly, the heterozygous animal A2420, typed as DH22B/DH7A, responded to the three peptides, VP2[74–88], VP4[20–34] and VP1[35–53], with decreasing magnitude. Heterozygous animal A2351, typed as DH22H/DH18A, responded strongest to VP2[74–88] due to the presence of the DH22H haplotype. The responses to the VP1 and VP4 peptides were more or less equally high, due to the presence of the haplotypes DH18A and DH22H, respectively.

For refinement of the recognition of the VP4 T-cell site by the different animals, a panel of 10 overlapping peptides, each 15 amino acids long, covering residues 17–40 of VP4, were tested using PBMC from animals A1983 (DH7A), A2399 (DH8A) and A2362 (DH18A/DH24A). Unfortunately, we were not able to use PBMC from animal A2346, which excluded analysis in the context of DH22B. The results are presented in Table 5. PBMC from animal A1983 responded to six of the peptides, covering amino acid sequence 17–36. Peptides covering amino acid sequence 20–39 elicited the strongest proliferation of PBMC from animal A2399. High responses to all peptides, with the exception of VP4[26–40], were found with PBMC from animal A2362. Since it was shown that none of these peptide-specific responses was likely to occur via the DH24A haplotype, DH18A must have been the haplotype responsible for this effect. Deduced from these results, three shorter peptides were synthesized and tested. As expected, VP4[22–31] was recognized in association with haplotypes DH7A and DH18A, and VP4[25–34] was recognized in association with haplotypes DH8A and DH18A. However, the 7-mer VP4[25–31] was not recognized.

Cytokine production by the PBMC following peptide-specific stimulation was measured after 24, 48 and 72 hr incubation with no peptide or one concentration of each peptide. The cytokine assays were performed using PBMC from the three homozygous cattle (A1983, A2399 and A2346), which proliferated to one or two of the three peptides (Table 4). Results of the IFN-γ assay are shown in Table 6. IFN-γ was found in all supernatants. However, a significantly higher concentration of IFN-γ was present in 24-, 48- and 72-hr supernatants of cells that specifically proliferated to peptide, as shown by enhanced [^{3}H]thymidine incorporation after 5 days of incubation. The presence of transcripts for IL-2 and IL-4 was tested by PCR using specific primers. In all 24- and 72-hr samples, IL-2 mRNA could be detected easily (data not shown). IL-4 mRNA was not detected in any sample of cells specifically proliferating to peptide. IL-4 mRNA was only

Table 5. Proliferation of PBMC to a panel of overlapping VP4 peptides

Animal no.	A1983	A2399	A2362
DH	7A/7A	8A/8A	24A/18A
BG*	565	588	589
FMDV-antigen†	+ + +	+ +	+ + +
VP4 peptides			
17–31‡	+ + +§	–	+ + +
18–32	+ + +	–	+ + +
19–33	+ + +	+	+ + +
20–34	+ + +	+ +	+ + +
21–35	+ + +	+ + +	+ + +
22–36	+ +	+ + +	+ + +
23–37	–	+ + +	+ + +
24–38	–	+ + +	+ + +
25–39	–	+ +	+ +
26–40	–	+	–
22–31	+ +	–	+ + +
25–34	–	+ +	+ +
25–31	–	–	–

* BG, background proliferation of PBMC presented as c.p.m. The value obtained is the mean of 12 cultures.

† Proliferation to 2·5 μg/ml purified FMDV strain A_{10}Holland.

‡ Proliferation to 5×10^{-5} M of the VP4 peptides. Peptide sequence is indicated by first and last amino acid of the VP4 amino acid sequence.

§ + + + = SI > 100; + + = 10 < SI < 100; + = 3·0 < SI < 10; – = SI < 3·0. (SI = stimulation index). The standard deviation of mean was 20% or less.

Table 6. Proliferation, IFN-γ secretion and detection of IL-4 mRNA by or in PBMC incubated with no peptide or with each peptide, representing one of the three FMDV T-cell sites

		SI*	Relative IFN-γ conc.†			IL-4 mRNA
Incubation time (hr)		114	24	48	72	48/72
A1983‡	No peptide	1·0	1·0	1·0	1·0	+
(DH7A/DH7A)	VP1[35–53]§	**3·1**	**1·8**	**1·4**	**1·4**	–
	VP2[74–88]	1·0	1·0	1·0	1·0	+
	VP4[20–34]	**19·3**	**3·4**	**4·7**	**3·1**	–
A2399	No peptide	1·0	1·0	1·0	1·0	+
(DH8A/DH8A)	VP1[35–53]	1·0	1·0	0·9	1·0	+
	VP2[74–88]	1·0	1·0	1·1	1·0	+
	VP4[20–34]	**7·8**	**2·4**	**3·9**	**3·5**	–
A2346	No peptide	1·0	1·0	1·0	1·0	+
(DH22B/DH22B)	VP1[35–53]	1·3	1·2	1·2	1·0	+
	VP2[74–88]	**22·2**	**2·7**	**3·5**	**3·1**	–
	VP4[20–34]	**3·4**	**2·3**	**2·6**	**3·2**	–

* Proliferation of cells was measured by a 5-day proliferation assay. SI (stimulation indices) are shown.

† Eight times diluted supernatants of cells were tested in an IFN-γ ELISA kit. Relative IFN-γ concentration was calculated as the ratio of the OD_{450} value obtained from the supernatant and the OD_{450} value obtained from the supernatant of the same cells incubated without peptide.

‡ Animal from which PBMC were tested with, between parentheses, the MHC class II haplotype (DH) as shown in Table 2.

§ PBMC were incubated with 10^{-6} M of the peptides indicated.

Bold numbers indicate SI > 3·0, OD_{450} ratios > 1·3 and samples in which no IL-4 mRNA was detected.

clearly detectable in 48- and 72-hr samples derived from cells proliferating non-specifically (Table 6). The enhanced IFN-γ secretion and no or decreased IL-4 transcription by cells specifically proliferating to the peptides indicated a shift towards a T-helper type-1 (Th1) type of response.

DISCUSSION

An attractive approach in vaccine development is the generation of vaccines based on synthetic peptides. However, since it is known that T cells recognize antigenic peptides only in association with products of the polymorphic MHC,[4] such a peptide vaccine may be effective only in a selected group of individuals. Here we studied the effect of bovine MHC class II polymorphism on the proliferative and cytokine responses to three T-cell sites of FMDV.

To refer to the complete MHC class II haplotype, we used the D haplotype (DH), as defined by several typing techniques for both the DR and DQ regions (= Ds type + DRB3 PCR-RFLP type + DRBF-type + DQA-RFLP type + DQB-RFLP type). A clear correlation between the MHC class II haplotype and the response to a particular peptide was found, which is summarized in Table 7. Although Holstein–Friesians were used throughout this study, D haplotypes 7A, 8A, 18A and 22B have also been found in other breeds of cattle.[24] So the three peptides are likely to be effective in other breeds of cattle as well. It is not certain whether the recognition of VP4[20–34] is possible in the context of the DH22H haplotype. This haplotype was only present in a heterozygous animal (A2351) that also carried the DH18A haplotype, which itself is responsible for a response towards this peptide. However, VP4[20–34] is recognized in association with the DH22B haplotype (animal A2346) which is, with respect to DR type, identical to the DH22H haplotype. Furthermore, the magnitude of the response to VP4[20–34] by PBMC from animal A2351 was clearly enhanced compared to the response to this peptide in the context of the DH18A haplotype only (as shown by animal A2362). Based on both observations, we suggest that VP4[20–34] might also be recognized in the context of the DH22H haplotype.

The recognition of this VP4 T-cell site in the context of the DH7A, DH8A and DH18A haplotypes was studied in more detail. A difference in the 'core' sequences was observed for

Table 7. Association between the T-cell sites recognized by PBMC from FMDV-vaccinated cattle and their MHC class II haplotype (DH) or associated DRB3-PCR-RFLP type

DH	DRB3-PCR-RFLP	VP1[35–53]	VP2[74–88]	VP4[20–34]
7A	7	+	–	+
8A	8	–	–	+
18A	18	+	–	+
22B	22	–	+	+
22H	22	–	+	+ ?
24A	24	–	–	–

recognition in the context of these different haplotypes: amino acid 22 (to 31 or less) of VP4 was essential for recognition in the context of the DH7A haplotype: for the DH8A haplotype, amino acid 33 (from 26 or more) was required, although responses increased dramatically as soon as amino acid 34 was included; and finally, in the context of the DH18A haplotype, the critical sequence seemed to include amino acid 25 (to 31 or less). However, the 7-mer VP4[25–31] was not recognized in the context of the DH18A haplotype. Apparently this relatively short peptide is much less effective in MHC binding and/or T-cell activation, as has been found in many other studies on T-cell responses to truncated peptides.[28,29] Another possibility is that in the context of the DH18A haplotype more than one 'core' sequence within the VP4 sequence 17–39 is present, one within region 22–31 and the other within region 25–34, each binding to the same or to different MHC class II molecules (e.g. DR and DQ). These kinds of shifts in 'core' sequences within one promiscuous T-cell site of an antigen have been described before.[30] Analysis of such a T-cell site indicated that the peptide assumed different conformations upon interaction with different MHC molecules.[31]

We did not observe a significant shift in cytokine profiles of PBMC that were derived from animals carrying different MHC class II haplotypes, but that proliferated to the same peptide. Similarly, no differences in cytokine profiles of PBMC that were derived from a single animal but that specifically proliferated to different peptides could be found. PBMC that specifically proliferated to peptide were all found to secrete more IFN-γ and less IL-4 than non-specifically proliferating cells, indicating a shift towards T-helper cells of the Th1 subtype upon peptide-specific stimulation.[7]

We do not know whether the responses to the FMDV peptides, reported here, are restricted via DR, DQ or maybe even other products expressed from the bovine MHC class II region.[32,33] Evidence of DR and/or DQ restriction might be obtained by testing other MHC-typed cattle matched either for DR or DQ. However, selection of such animals will not be easy, since DQ and DR are in strong linkage disequilibrium.[34,35] Alternatively, the use of blocking DR- and DQ-specific monoclonal antibodies (mAb) or direct MHC–peptide binding assays could be informative in this matter. However, well-defined mAb are currently unavailable for these studies.

Knowledge of the precise restriction element is not necessarily essential for the prediction of the effectiveness of a peptide in a MHC-typed animal. Cross-overs between the different MHC class II loci appear to be limited, leading to a strong linkage between DR, DQ and probably also other products.[34,35] Thus, in general, typing for only one MHC class II product might be sufficient to predict the outcome of the response towards a peptide. Although about 600 animals of the WAU herd are typed for DRB3 by PCR-RFLP, only a few animals are typed for the complete MHC class II haplotype. If we consider our findings for this population of 600 animals, the following predictions can be made with regard to the successful implementation of peptide vaccines (for this purpose the DRB3-PCR-RFLP types, associated with the DH haplotypes studied so far, are included in Table 7). Complete non-responsiveness to the three FMDV peptides was observed for the DH24A haplotype. Seventeen animals in the typed WAU herd were found to be homozygous for DRB3*24, a DRB3 type which is found to occur exclusively in the DH24A haplotype. Thus, only 3% of non-responders from the total herd of 600 animals would be expected, on the basis of the frequency of the DH24A haplotype. To date, 32 DRB3-PCR-RFLP types have been defined. So far, four of these have been shown to be associated with a response to VP4[20–34]. Yet, 60% of the WAU herd was positive for one or two of these four responder DRB3-PCR-RFLP types. If the distribution of class II haplotypes is representative for the Holstein–Friesian breed in general, VP4[20–34] would be a good candidate for inclusion in a peptide vaccine, especially since this site is derived from a highly conserved region of the virus as well. The amino acid sequence of VP4[20–34] is 100% identical between serotypes A, O and C of FMDV. An effective synthetic vaccine might be generated for example by coupling the VP4 T-cell site in combination with one or more B-cell epitopes to the lipotripeptide (P_3CSS), as studied by Wiesmuller *et al.*[36] or by inclusion of the peptides into liposomes.[37]

In conclusion, these findings show for the first time that a strong MHC-restricted selection of T-cell epitopes also occurs in an outbred population like cattle. This will have major implications for research on vaccine development, and for the design of synthetic vaccines in particular. The influence of MHC polymorphism may be less apparent in the response to complete viral proteins. Still, the effect of MHC polymorphism must not be under-estimated when testing new subunit vaccines in randomly chosen groups of animals. In order to establish the efficacy of the vaccine in different MHC backgrounds, the animals used should be typed in advance. Then groups of animals can be selected in which the most frequent MHC class II types are represented and equally distributed. For the development of a peptide vaccine, MHC polymorphism will surely have an influence, as we have shown here. In order to design a peptide vaccine which is effective in all animals, the response to different T-cell epitopes should be tested for all possible MHC haplotypes. For this kind of study homozygous animals are indispensable. Finally, a combination of a limited number of peptides, covering a T-cell response in association with all haplotypes should be included in the vaccine. The present study shows that for this purpose conserved and promiscuous epitopes of a complex pathogen like FMDV can be found that largely circumvent the MHC-related restriction even in an outbred population.

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