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Bovine T cells recognize antigen in association with MHC class II haplotypes defined by one-dimensional isoelectric focusing

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SUMMARY

A recently established, one-dimensional isoelectric focusing (IEF) method for distinguishing major histocompatibility complex (MHC) class II polymorphisms in an outbred species, cattle, has allowed us to analyse the involvement of the MHC in the recognition of antigen by bovine T cells. Bovine T-cell lines of Th cell phenotype (BoCD4$^+$) specific for ovalbumin were generated from six individual high responder animals. These animals were bovine MHC (BoLA) class II typed using the IEF technique which detects bovine DR-like products. Four of the animals were shown to be heterozygous and two were homozygous for the IEF specificities. Six out of the 13 IEF specificities (EDF types) detected so far were represented by this group of animals. The cell lines were tested against a panel of IEF-typed antigen-presenting cells (APC) from unrelated donors. The lines only responded to antigen in proliferation assays when the APC shared at least one MHC class II EDF specificity with the BoCD4$^+$ cell line. The responses did not correlate with BoLA class I specificities. However, lines from one of the animals were consistently generated to one of the two haplotypes only. This suggests that there are non-responder alleles to a multi-epitope antigen, present in the cattle population. The results demonstrate that IEF of bovine MHC class II products defines haplotypes of functional relevance, and may indeed be identifying the actual restriction elements involved in presentation of ovalbumin. These results have important implications for future vaccine design in an outbred species, particularly in terms of immune response gene effects and disease associations.

INTRODUCTION

To design subunit vaccines consideration of major histocompatibility complex (MHC) Ir gene effects in the target species is essential. MHC class II molecules are highly polymorphic heterodimeric glycoproteins. T helper (Th) cells recognize foreign antigen in association with self-MHC class II molecules on the surface of antigen-presenting cells (APC).[1] MHC class II restriction was first described in guinea-pigs[2] and has been extensively reported in several other species, including mouse[3] and man.[4] However, more limited information on bovine MHC (BoLA) class II antigens is currently available. The MHC class II region was first defined in cattle as the major locus controlling mixed lymphocyte reactions (MLR) and, in families at least, these specificities were clearly linked to BoLA class I haplotypes.[5,6] However, no suitable typing method for BoLA class II haplotypes was available[7] until recently. Restriction fragment length polymorphism (RFLP) analysis of bovine DNA, using human class II probes, has shown that there are several DQ-like and DR-like genes with considerable polymorphisms present.[8] The DQ and DR regions appear to be in strong linkage disequilibrium with approximately 30 DQ-DR haplotypes detected.[9] However, it is not clear how many loci and alleles are expressed as products and function as restriction elements. A one-dimensional isoelectric focusing (IEF) technique for typing DR-like BoLA class II products has now been reported.[10,11] Using this method, the former group, in collaboration with our laboratory, could identify, in Dutch and British Friesians, 12 different BoLA class II products.[10] Each IEF-defined haplotype is associated with a distinct set of RFLP-defined DQ-DR haplotypes.[12] Thus it is now possible to investigate functional aspects of these MHC class II haplotypes. We have shown that bovine Th responses are dependent upon MHC class II-positive APC.[13] We have also shown that variation in T-cell response to a multi-epitope molecule, ovalbumin, correlates with BoLA class I haplotypes in half-sib families, implying correlation with BoLA class II haplotypes.[14] More recently we have generated antigen-specific bovine cell lines with Th cell phenotype which appear to be depleted of alloreactivity.[15] Using the IEF method together with these lines we demonstrate here that the IEF method detects functionally relevant specificities and that bovine CD4$^+$ (BoCD4$^+$) cells are BoLA class II haplotype restricted. These results are of fundamental importance for our

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studies on vaccine design for cattle but are also of relevance to all outbred species.

MATERIALS AND METHODS

Animals

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

Immunization

The animals were immunized with 500 μg of ovalbumin (type VII; Sigma, Poole, Dorset, U.K.) 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.

Source of IL-2

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

Preparation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected and PBMC were separated on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) as previously described.[13] Blood samples were taken just before immunization and at varying times thereafter.

Generation of antigen specific BoCD4$^+$ cell lines

The generation and growth requirements of these lines is described elsewhere[15] and are briefly as follows.

PBMC from animals immunized with ovalbumin which gave high responses in antigen-dependent proliferation assays[14] were selected for the generation of BoCD4$^+$ lines, following the method of Kimoto & Fathman.[16] After 7 days culture in the presence of ovalbumin, a blast-enriched population was obtained from the proliferating PBMC by separation on a discontinuous Percoll gradient.[17] 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. The phenotype and specificity of these cell lines were similar to those described previously.[15] 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).[18] They required autologous MHC class II-positive APC for antigen-specific proliferation, which was inhibitable by anti-bovine MHC class II mAb but not by an anti-bovine MHC class I mAb.[15] Background counts were usually less than 200 c.p.m. and the optimal dose of ovalbumin was 20 μg/well, which was the concentration employed in the experiments reported here.

Proliferation assay

This was essentially as described elsewhere[15] and is briefly as

Table 1. IEF phenotyping of cell panel

	BoLA			BoLA	
APC donor	Class II EDF type	Class I type	APC donor	Class II EDF type	Class I type
1[defg]	6, 7	w11, w18	20[e]	6, 9	w10, w17
2[defg]	4, 6	w10, w15	21	8, 13	w14, w18
3[defg]	7, —	w11, —	22[abc]	4, 13	w15, w19
4[abcg]	13, —	w18, —	23	4, 13	w10, w18
5[abcg]	2, 9	w6, Ed99	24[abc]	3, 4	w11, w17
6[abcg]	2, 13	w18, —	25[be]	9, 13	w14, w18
7	4, 6	w10, w14	26	13, —	w10, w18
8	7, 12	w11, w14	27	6, 7	w10, w11
9[ab]	4, 5	w15, w32	28[adf]	8, 11	w14, w18
10	7, —	w17, w19	29[bd]	2, 7	w3, w13
11[c]	6, 8	w10, w14	30[ac]	5, 11	w18, —
12[e]	4, 6	w18, —	31[c]	5, 6	w10, w32
13[ef]	4, 6	w10, w15	32[a]	2, 13	w3, w14
14[be]	1, 7	w13, Ed104	33[abcd]	2, 3	w20, Ed99
15[e]	3, 4	w10, w20	34	4, 7	w8, w11
16	7, 8	w11, w14	35[a]	2, 9	w2, Ed99
17	1, 7	w11, w14	36[abcd]	2, 5	w32, Ed99
18[e]	4, 7	w11, w15	37	2, 6	w3, w10
19[abc]	1, 8	w14, —	38[cd]	2, 6	w14, Ed99

[a] Cell line 1 did not respond to these APC.
[b] Cell line 2 did not respond to these APC.
[c] Cell line 3 did not respond to these APC.
[d] Cell line 4 did not respond to these APC.
[e] Cell line 5 did not respond to these APC.
[f] Cell line 6 did not respond to these APC.
[g] Antigen specific cell lines were prepared from these donors.

follows. Blasts (1×10^4/well) and irradiated PBMC (6×10^4/well) (5000 rads) were incubated together with ovalbumin (20 μg/well) and cell proliferation measured after 72 h by a final 6-h 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.*[5] was used to detect all of the internationally agreed workshop specificities.[19] Other sera detecting specificities not yet agreed internationally were given an Ed prefix. All specificities behaved as alleles of a single class I hocus.

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

IEF and immunoprecipitation of BoLA class II antigens was carried out according to the method of Joosten *et al.*[10] using a rabbit anti-human HLA-DR antisera which precipitates BoLA class II molecules. Twelve distinct banding patterns for the β-chain were obtained, with two bands per haplotype; α-chains appeared to be non-polymorphic. Since then we have detected another haplotype and we designate the patterns as EDF1–

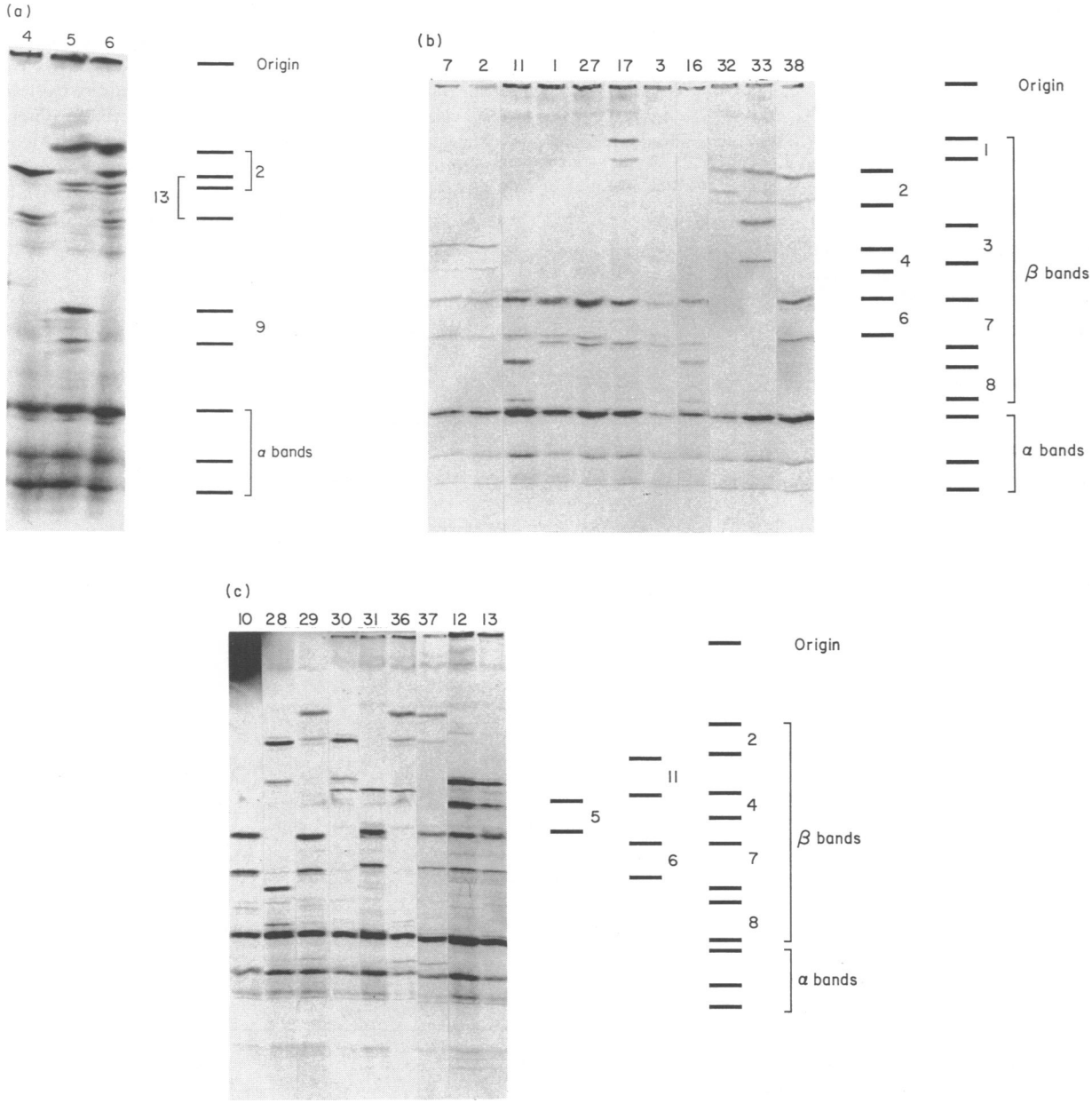

Figure 1. 1D-IEF analysis of BoLA class II products from PBMC from 25/38 donors as described in Table 1. The left hand side shows the autoradiographs and the right hand side shows the interpretative drawings prepared from them. The characteristic banding patterns were designated as shown, based on the findings of Joosten *et al.*[10] and R. A. Oliver and J. L. Williams (unpublished observations). They were given EDF types as indicated with two β bands per haplotype. The numbering for the lanes refers to the numbering of donors in Table 1.

EDF13 (E for Edinburgh, D in analogy to the human HLA class II system, and F for focusing).

Statistics

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

RESULTS

Bovine MHC (BoLA) class I and class II (EDF) haplotypes of the bovine CD4$^+$ antigen-specific cell lines

Six cell lines were generated, as described in the Materials and Methods, from six animals (1–6), as shown in Table 1. Their IEF patterns are shown in Fig. 1a,b. Four of the animals are heterozygous (1, 2, 5 and 6). Animals 3 and 4 appear to be homozygous for both BoLA class I (serological typing) (w11 and w18, respectively) and BoLA class II (EDF7 and EDF13, respectively), as assessed by IEF typing (Table 1 and Fig. 1a,b) and also by MLR (results not shown). Thus of the 13 EDF specificities detected so far, six are represented within this group. Animals 1, 2 and 3 are members of a half-sib family group. The other animals are unrelated. Within the family group, EDF7 is inherited together with BoLA w11 from the bull, by Animals 1 and 3. The bull's other haplotype is EDF6 and BoLA w10 and is inherited by Animal 2 (the EDF6 in Animal 1 is inherited from the dam together with BoLA w18).

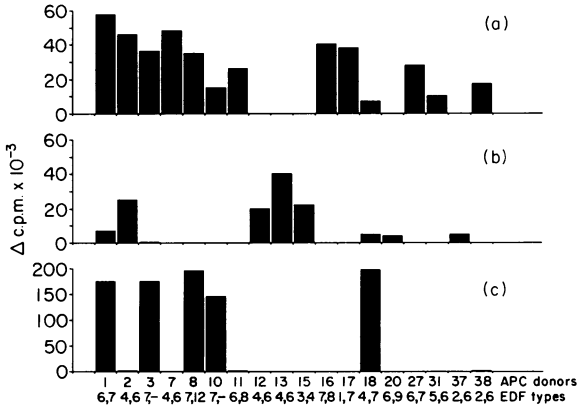

Figure 2. Proliferative responses by cell lines 1, 2 and 3 (a, b and c respectively) to a panel of APC (irradiated PBMC) described in Table 1. Δc.p.m. = c.p.m. in the presence of antigen − c.p.m. with medium alone. Responses = mean of quadruplicate cultures. SD were always less than 10%. No response was obtained by any of these cell lines to a further selection of APC with non-shared EDF types, as described in Table 1.

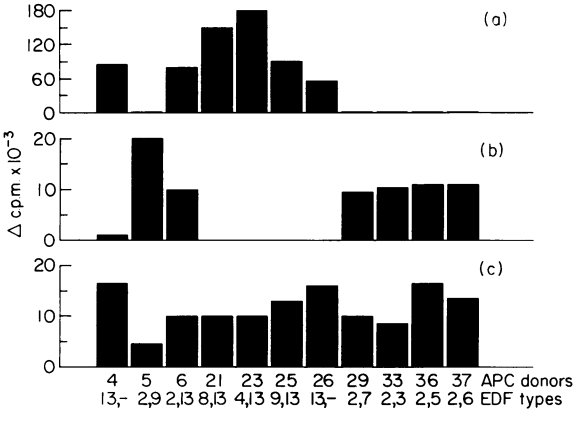

Figure 3. Proliferative responses by cell lines 4, 5 and 6 (a, b and c, respectively). Details as in Fig. 1.

MHC class II haplotype restriction of BoCD4$^+$ antigen-specific cell lines

Proliferation of the six cell lines to ovalbumin in the presence of self-irradiated PBMC as the source of APC was highly significant ($P < 0.01$), although the magnitude of the response varied between animals.

The six cell lines were analysed for responsiveness with a panel of 38 BoLA class I and IEF class II typed APC, as shown in Table 1. The IEF patterns of 25/38 APC donors are shown in Fig. 1, which includes 11/13 EDF types. All of the 13 EDF specificities are represented in the panel, except EDF10 which is extremely rare in our population (R. A. Oliver, unpublished observations). The donors are all unrelated except for the first three which are members of a half-sib family group. The response patterns are shown in Figs 2 and 3. None of the lines responded to any APC in the absence of antigen, and background levels were usually less than 200 c.p.m. (results not shown). Although the degree of response varied with different APC, clear-cut restriction patterns were observed with all six cell lines. All of the positive reactions with each cell line were highly significantly different from the control ($P < 0.001$). Positive responses by the six cell lines were only observed with APC with EDF types in common with the individual cell line (Figs 2 and 3). The responses by cell lines 1–3 (Fig. 2) from the half-sib family had some common reactivities corresponding to the EDF types 6 and 7 inherited from the sire. Similarly, in Fig. 3 the donors for cell lines 4–6 shared EDF types (2 and 13), although in this case they were unrelated. However, the response patterns again reflected the IEF typing for the APC. The patterns of non-responsiveness by each cell to individual APC are indicated in Table 1. In each case shown, APC expressing non-shared EDF types were unable to present antigen. The only exception was EDF9, in that cell line 5 (typed as EDF 2,9) did not respond to APC from donors 20 and 25, yet both expressed EDF9.

The patterns of response also indicated that the cell lines were not responding to BoLA class I haplotypes. For instance, line 1 did not respond to APC from donors 4, 6, 28 or 30, all of which shared the BoLA w18 haplotype but neither EDF6 nor EDF7 with animal 1; lines 1 and 3 responded to APC from donor 10, which expressed a shared class II haplotype, EDF7, but did not have BoLA class I haplotypes in common.

DISCUSSION

This paper demonstrates that the IEF technique defines bovine MHC class II haplotypes with functional relevance for antigen presentation and possibly also for Ir gene effects. The results clearly show that APC from IEF-typed unrelated donors can only present antigen if they share at least one EDF specificity with the responding cell line. No correlation between response and BoLA class I haplotypes was observed. This confirms our previous results which indicated that mAb specific for bovine

MHC class II molecules inhibited T-cell line proliferation, whereas mAb specific for bovine MHC class I molecules did not.[15]

Dependence on MHC class II products by bovine T cells has been demonstrated using mAb specific for non-polymorphic bovine MHC class II determinants.[14,20,21] However, the extent and role of polymorphism in the recognition of antigen was unknown. The IEF method together with BoCD4+ antigen-specific cell lines depleted of alloreactivity has made the analysis of bovine MHC class II haplotype restriction possible. Although these results do not prove identity between the IEF product and the restriction element recognized by the cell lines, the IEF patterns can be used to indicate functionally relevant MHC class II haplotypes.

The very close correlation between IEF patterns and response is perhaps surprising and indicates that either that the cell lines were restricted to the IEF-defined product or that they are restricted to products from an extremely closely linked locus. The IEF method appears to detect a single BoLA class II subregion,[10] probably analogous to the human HLA-DR region. At the DNA level there appears to be at least one bovine DR-like and one bovine DQ-like locus,[22,23] both of which are polymorphic but very closely linked.[8] Cloning of the BoCD4+ antigen-specific lines and RFLP analysis of both lines and APC may help to clarify these issues. With HLA haplotypes, the MHC class II alleles defined by 1D-IEF using locus specific mAb correlate well with previously defined serological and cellular specificities.[24] This suggests that if equivalent mAb were available for cattle which could differentiate between bovine DR and DQ products, further definition of restriction elements would be possible.

These experiments would have proved difficult, if not impossible, if the lines had not been depleted of alloreactivity.[15] This is unlike the situation with ovalbumin-specific ovine cell lines,[25] where cross-reactivity with allo APC in the absence of antigen was observed. Such cross-reactions have also been observed in other species.[26]

Of the six haplotypes examined only one failed to show any reactivity—EDF9. The most likely explanations are either that EDF9 represents a non-responder haplotype with the EDF9 molecule having low affinity for the relevant ovalbumin peptide, as has been demonstrated for non-responders in other species,[27] or that the line recognized another class II molecule not detected by the IEF method. Indeed there was a striking difference in response to self-APC compared to EDF2 typed APC by line 5.

Other than in its extreme case, no obvious bias towards one or other BoLA class II haplotypes was observed with the other three heterozygous lines. This is unlike the apparent biases observed with bovine MHC class I-restricted *Theileria parva*-specific Tc.[28] However, the magnitude of response varied both between lines and also between different APC with the same EDF type. The former may reflect different T-cell repertoires, possibly influenced by MHC type. The variation in response elicited by APC with apparently identical EDF types may have depended on the quantity of MHC class II expressed on the APC surface. Without relevant haplotype-specific antisera we are as yet unable to confirm this. However, this cannot have been the only reason since the patterns of response to specific APC varied between lines, e.g. cell lines 1 and 3 both responded to APC sharing EDF7 yet cell line 1 had a low (though significant) response to APC 10 and 18 compared to APC 8, whereas cell line 3 responded equally well to all three APC. Further studies using cloned lines are planned.

In conclusion the reactivities of the BoCD4+ antigen-specific T-cell lines indicate that the IEF method detects functionally relevant haplotypes. Therefore it should prove a highly sensitive method for detecting potential bovine MHC–disease associations, for defining fine specificities of BoCD4+ cell lines and clones, and also for further investigations of bovine Ir genes. All of these are important considerations for the design of future vaccines.

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