Short paper

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Isoelectric focusing of bovine major histocompatibility complex class II molecules*

Serological approaches have been relatively unsuccessful in defining the allelic products of the bovine major histocompatibility (MHC) class II loci. We demonstrate that bovine class II allelic products can be characterized by precipitation with a polyclonal antiserum and separation using one-dimensional isoelectric focusing. Polymorphic β chains were present in immunoprecipitates from both biosynthetically and surface-labeled lectin-stimulated bovine T cells. Precipitates from biosynthetically labeled but not surface-labeled T cells contained a basic invariant chain and a non-polymorphic structure. The non-polymorphic structure appears to be a β chain. The polymorphic class II β chain co-segregated with bovine MHC class I allelic products in a half-sibling family, providing evidence for linkage between bovine class I and class II loci. This approach to the biochemical analysis of the bovine class II structures should facilitate the investigation of the association between the bovine products and disease susceptibility.

1 Introduction

The major histocompatibility complex (MHC) loci comprise the most polymorphic gene region known in mammals. The class II products of the MHC loci are transmembrane glycoproteins which play a crucial role in antigen presentation [1]. Susceptibility to a number of diseases is closely associated with certain MHC phenotypes in man [2, 3]; there is also evidence that the same is true in cattle [4]. Because of breeding practices, large half-sibling cattle families are available for study. Cattle, therefore, provide an excellent model for assessing the relationship between disease susceptibility and the MHC. However, the definitive demonstration of the relationship between MHC phenotype and disease susceptibility in cattle will require a precise definition of the bovine MHC allelic products.

Limited biochemical information is available at the present time about bovine MHC class II molecules. These molecules appear to be heterodimers and are homologous to their human and mouse counterparts [5]. It has been proposed that at least two separate loci encode class II molecules that are involved in the mixed lymphocyte reaction (MLR) in cattle [6]. Although alloreactive T lymphocyte clones have been used to type animals at their class II loci [7], progress in serotyping of animals at these loci has been limited. MHC analysis at the DNA level using class II-specific mouse and human DNA probes has revealed extensive polymorphism at the bovine loci encoding class II α and β chains [8–10].

In the present studies, we demonstrate that bovine class II allelic products can be characterized by precipitation with a polyclonal antiserum and separation using one-dimensional isoelectric focusing (1D IEF).

2 Materials and methods

2.1 Animals and bovine lymphocyte antigen (BoLA) typing

Heparinized blood was obtained by venipuncture from several different breeds of cattle. The BoLA-A genot types, breeds and the relationships of these animals are shown in Table 1. Peripheral blood lymphocytes (PBL) were tested for BoLA-A class I antigens [11] using a standard lymphocyte microcytotoxicity test [12].

2.2 Cell culture and radiolabeling

PBL from cattle were cultured and either biosynthetically or surface labeled as described previously [13]. Radiolabeled cells were lysed, lysates were precleared, and class II molecules were precipitated and sialidase treated as described previously [13].

2.3 Antibodies

Rabbit antiserum directed against human class II molecules [14], provided by H. Ploegh (Netherlands Cancer Institute, Amsterdam, The Netherlands), was used to precipitate bovine class II structures. Normal rabbit serum (Sigma, St. Louis, MO) was used as negative control antibodies.

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Abbreviations: **BoLA:** Bovine lymphocyte antigen **1D IEF:** One-dimensional isoelectric focusing **MHC:** Major histocompatibility complex **PBL:** Peripheral blood lymphocytes **RFLP:** Restriction fragment length polymorphism **SDS-PAGE:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis **Sialidase:** EC 3.2.1.18 (Neuraminidase)

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Table 1. BoLA-A assignments of the cattle used in this study

Animal #	Tag	BoLA-A	Breed	Relationship
6	4756	UR6.4/UR6.4	Holstein-Friesian	Unrelated
8	4436	UR6.4/UR6.4	Holstein-Friesian	Unrelated
9	4754	W12/W8.1	Holstein-Friesian	Unrelated
10	4601	W12/W12	Holstein-Friesian	Unrelated
11	4664	W12/W12	Holstein-Friesian	Unrelated
12	4605	W8.1/"Blank"	Holstein-Friesian	Unrelated
13	4780	W20/W20	Holstein-Friesian	Paternal[a)] half-sib
14	4672	W20/W20	Holstein-Friesian	Paternal[a)] half-sib
15	4771	W20/"Blank"	Holstein-Friesian	Paternal[a)] half-sib
16	4701	W20/W20	Holstein-Friesian	Paternal[a)] half-sib

a) Animals 13–16 all have the same father (BoLA-A type of father was W20/W8.1).

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were resuspended in SDS-PAGE sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 0.0625 M Tris-HCl, pH 6.8, 0.01% bromophenol blue) and analyzed using the discontinuous method of Laemmli [15], with a 10% separating gel and a 3% stacking gel.

2.5 1D IEF and 2D IEF

1D IEF was carried out as described previously [13]. Gels were poured with the following ampholine content: 3% pH 5–7, 1.4% pH 3.5–10, and 1.0% pH 7–9. 2D gel electrophoresis was carried out using the method of O'Farrell [16] with IEF in the first dimension and SDS-PAGE in the second.

3 Results

3.1 Characterization of bovine MHC class II allelic products

To characterize bovine MHC class II allelic products, it was first necessary to identify anti-class II antibodies which recognize phylogenetically conserved monomorphic epitopes on bovine class II molecules. SDS-PAGE analysis was performed on surface and biosynthetically labeled bovine class II structures immunoprecipitated from lysates of lectin-stimulated PBL with a rabbit polyclonal antiserum generated against human class II molecules. The PBL were from animal #8 (4436, a BoLA-A locus homozygote; Table 1). A single class II light (β) chain was precipitated (Fig. 1a). Heavy (α) chains were not evident on these SDS-PAGE gels, consistent with the known weak reactivity of this antiserum with bovine α chains (data not shown).

These immunoprecipitated BoLA class II glycoproteins were then separated on the basis of their charge using 1D IEF (Fig. 1b). This allowed us to identify the different class II allelic products present on bovine T lymphocytes. Since class II molecules from most species are sialic acid-containing glycoproteins, immunoprecipitates were treated with sialidase to remove negatively charged sialic acid residues from these class II structures. Differences in the IEF banding pattern of the various sialidase-treated allelic products, therefore, should not reflect charge modification due to sialilation, but rather should reflect actual differences in the amino acid sequences of the class II structures. Sometimes, however, sialidase digestion can be incomplete, resulting in an array of two or more bands for each class II allelic product. This can be recognized in sialidase-treated precipitates when compared with untreated precipitates by the reduction in intensity of the more acidic bands and a concomitant increase in intensity of the more basic bands. The sialilated class II β light chains focused at the acidic end of the gel. After sialidase treatment, each β chain is represented on the gel by two bands, probably the unsialilated molecule and the β chain modified with one sialic acid. (It is, however, possible that two translation products are made from

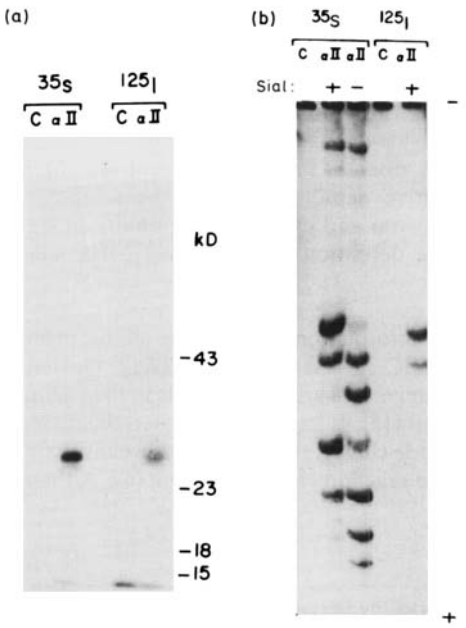

Figure 1. (a) Rabbit antibodies generated against human class II glycoproteins precipitate bovine class II structures. Lectin-stimulated PBL from animal #8 were biosynthetically ^{35}S or surface ^{125}I-labeled, lysed, precleared and class II glycoproteins were immunoprecipitated with rabbit antiserum directed against human class II antigens (α II). Lysates were also incubated with normal rabbit serum (C). Sialidase-treated class II glycoproteins were analyzed on a discontinuous 10% SDS-PAGE gel. The mobilities of the standards (43, 23, 18 and 15 kDa) are indicated. (b) Bovine class II glycoproteins are sialilated. 1D IEF analysis of bovine class II glycoproteins. Samples were identical to those described in (a) and were analyzed by 1D IEF. Samples were either treated with sialidase (+) or left untreated (−). Samples were subjected to 1D IEF with the anode at the bottom of the gel. The pH gradient across the gel was approximately pH 4–8.

each β chain gene after transcription and splicing.) The most basic band was not sialilated.

Since we were interested in identifying functional, surface-expressed bovine class II structures, we also surface-labeled lectin-stimulated PBL from animal #8 and these immunoprecipitates were analyzed by 1D IEF (Fig. 1b). The only surface-expressed molecule that was labeled with iodine was a single sialilated light chain (Fig. 1b). ^{125}I failed to label the more acidic of the two sialilated sets of bands, suggesting that it may not be surface expressed. The most basic of the focused molecules was also not labeled with iodine, suggesting that it, too, was not expressed on the cell surface. (Our inability to label these molecules could also result from their tyrosine residues being inaccessible to iodination.)

We then used SDS-PAGE to assess the M_r of the focused glycoproteins present on the IEF gels to determine whether they were indeed classical MHC class II allelic products. The two more basic chains of animal #8 were identical in size, consistent with them being β chains (data not shown). The basic unsialilated focused band was shown to be identical in M_r to the more acidic β chains (data not shown).

3.2 Bovine MHC class II β chain polymorphism

Human class II loci are among the most polymorphic gene loci yet examined. It was, therefore, of interest to investigate polymorphism of these loci in cattle. To do this, we precipitated class II structures from biosynthetically labeled lysates from lectin-stimulated lymphocytes from BoLA-A locus-typed animals. Since we were interested in identifying sialilated class II glycoproteins, we examined both sialidase-treated and untreated precipitates. The most basic unsialilated band was shown to be non-polymorphic, suggesting that it is the bovine γ chain. Surprisingly, in this sample of BoLA-A-typed animals, one of the BoLA class II β chains was not polymorphic. Moreover, this same sialilated β chain was expressed by every PBL population studied from Holstein-Friesians and Brown-Swiss, as well as from Herefords (data not shown). Interestingly, these bands were not seen in lysates of surface-labeled bovine lymphocytes (Fig. 1b, and data not shown). The other more basic chains were, however, polymorphic (Fig. 2).

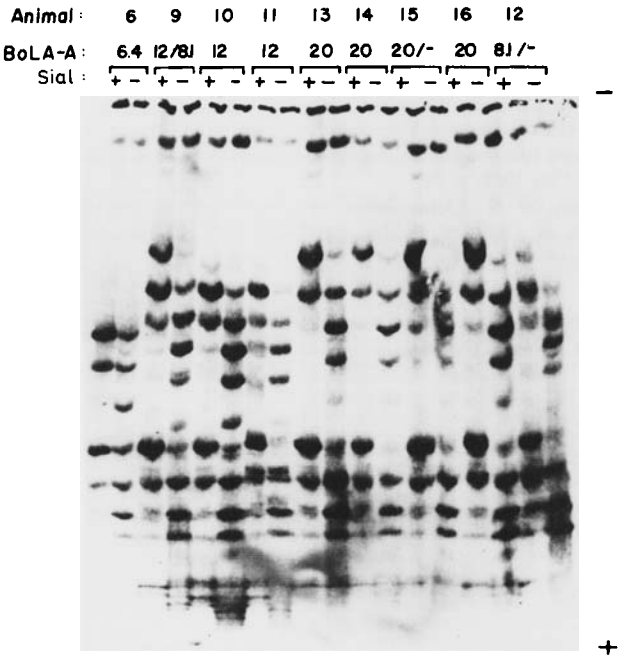

Figure 2. Cattle appear to have both polymorphic and non-polymorphic class II β chains. Class II structures were precipitated from lectin-stimulated PBL and were sialidase treated (+), or left untreated (−), and subjected to 1D IEF. The BoLA-A serological typing of the animals is shown. The anode is at the bottom of the gel and the pH gradient across the gel was approximately pH 4–8.

3.3 Linkage between bovine MHC class I and class II

There appeared to be evidence for linkage between certain class I serotypes and some of the polymorphic class II β chains (Fig. 2). Lymphocytes from animal #6 (BoLA-A UR6.4/UR6.4) expressed β chains that were only present on the surface of lymphocytes from the cattle which were BoLA-A UR6.4. Lymphocytes from the BoLA-A W12 homozygotes (animals #10 and #11) expressed identical class II β chains, and lymphocytes from the BoLA-A W12/W8.1 heterozygote (animal #9) also expressed this same β chain in addition to a different second β chain allelic product (Fig. 2). Another β chain allelic product was present on the surface of all the BoLA-A W20/W20 homozygotes (animals #13, #14 and #16, all paternal half sibs) and these animals appeared to be homozygous for this β chain allele. Lymphocytes from the BoLA-A W8.1 animal expressed the same allelic product as the BoLA-A W12/W8.1 heterozygote.

4 Discussion

The MHC is the major genetic region known to be associated with disease susceptibility. Due to the existence of large half-sibling families, cattle provide an important model to investigate any associations between the MHC and disease. Little biochemical information is presently available on bovine class II products. Traditional serologic techniques have not been successfully applied to the characterization of these bovine gene products and other attempted approaches do not allow their analysis at the molecular or population level. Thus, our demonstration that 1D IEF can be used in cattle to detect allelic products of class II β chain loci increases the likelihood that MHC class II-disease associations can be detected in cattle, if they indeed exist.

The non-polymorphic, very basic, unsialilated molecule defined in these studies probably is the bovine homolog of the γ chain. This highly basic invariant chain has been described in humans [17], rat [18], mouse [19], guinea pig [20] and the syrian hamster [21]. Preliminary evidence for the presence of the invariant chain in cattle has also been presented [5]. The chain appears to be associated with α/β class II heterodimers in the cytoplasm in humans ([22, 23]; Rudd and Crumpton, unpublished data).

Products of two β chain gene loci have been identified in these studies. These β chains have been identified on the basis of their more basic isoelectric point and lower M_r when compared to α chains. One of the β chains precipitated from the lysates of biosynthetically labeled lymphocytes was non-polymorphic. Cattle from all three breeds examined synthesize this class II β

chain. This β chain was not present in precipitates that were surface-labeled, suggesting either that it did not label well or was not expressed on the surface of the cell. Recently, Andersson [24] has presented evidence for a bovine class II β chain locus with only three alleles by restriction fragment length polymorphism (RFLP) analysis that he has called the DY β locus. Perhaps the non-polymorphic β chains evident on our gels are the products of this locus. It is also possible, however, that this non-polymorphic molecule could be either a modified γ chain or another invariant chain. In humans, for example, the very basic γ chain is processed by the addition of O-linked oligosaccharides and complex N-linked glycans [22, 23]. However, it is unlikely that this non-polymorphic bovine molecule is a processed γ chain since the more mature human γ chains are similar in size to the human DR heavy chains. The non-polymorphic bovine chain is the same M_r as the expressed bovine β chain. Finally, this non-polymorphic structure might be an α chain. Its acidic isoelectric point and non-polymorphic nature would be consistent with this.

The polymorphic β chains expressed by lectin-stimulated T cells appeared to co-segregate with BoLA-A serological specificities. This provides evidence that the BoLA class II loci and their class I counterparts are closely linked. Teale and Kemp [7] have also suggested that the bovine class I and class II loci are linked in studies of the reactivity of $CD4^+$ alloreactive clones in a family. Evidence for linkage has also been provided by Usinger et al. [25] using MLR studies in full-sib families, and Andersson et al. [8] using RFLP analysis.

IEF has proven to be highly sensitive in discriminating between MHC class II structures which differ only slightly in their amino acid makeup in several different species. Analysis of the DNA encoding the human MHC allelic products by RFLP has also proved useful in detecting polymorphism at the class II loci and has been readily applied in characterizing bovine MHC class II loci [8–10]. A difference between RFLP patterns of two cattle, however, does not necessarily mean that cells from these individuals express different class II MHC allelic products, since restriction enzymes also cut non-coding DNA. Thus, 1D IEF of precipitated glycoproteins complements RFLP analysis in that it facilitates the direct examination of the expressed class II allelic products.

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5 References

1 Kaufman, J. F., Auffray, C., Korman, A. J., Shackelford, D. A. and Strominger, J., *Cell* 1984. *36:* 1.
2 Todd, J. A., Bell, J. I. and McDevitt, H. O., *Nature* 1987. *329:* 599.
3 Sinha, A. A., Brautbar, C., Szafer, F., Friedmann, A., Tzfoni, E., Todd, J. A., Steinman, L. and McDevitt, H. O., *Science* 1988. *239:* 1026.
4 Lewin, H. A., Wu, M. C., Stewart, J. A. and Nolan, T. J., *Immunogenetics* 1988. *27:* 338.
5 Hoang-Xuan, M., Charron, D., Zilber, M.-T. and Levy, D., *Immunogenetics* 1982. *15:* 621.
6 Usinger, W. R., Curie-Cohen, M. and Stone, W. H., *Science* 1977. *196:* 1017.
7 Teale, A. J. and Kemp, S. J., *Anim. Genet.* 1987. *18:* 17.
8 Andersson, L., Bohme, J., Rask, L. and Peterson, P. A., *Anim. Genet.* 1986a. *17:* 95.
9 Andersson, L., Bohme, J., Peterson, P. A. and Rask, L., *Anim. Genet.* 1986b. *17:* 295.
10 Andersson, L. and Rask, L., *Immunogenetics* 1988. *27:* 110.
11 Bull, R. W., Lewin, H. A., Wu, M. C. et al., *Anim. Genet.* 1989. *20:* 47.
12 Terasaki, P. I., Bernoco, D., Park, M. S., Ozturk, G. and Iwaki, Y., *Am. J. Clin. Pathol.* 1978. *69:* 103.
13 Watkins, D. I., Kannagi, M., Stone, M. E. and Letvin, N. L., *Eur. J. Immunol.* 1988. *18:* 1425.
14 Neefjes, J. J., Hensen, E. J., DeKroon, T. I. P. and Ploegh, H. L., *Immunogenetics* 1986. *23:* 341.
15 Laemmli, U. K., *Nature* 1970. *227:* 680.
16 O'Farrell, P. H., *J. Biol. Chem.* 1975. *250:* 4007.
17 Charron, D. J. and McDevitt, H. O., *Proc. Natl. Acad. Sci. USA* 1979. *76:* 6567.
18 Frelinger, J. G., Hood, L. and Wettstein, P., *Transplant. Proc.* 1981. *13:* 1360.
19 Jones, P. P., Murphy, D. B., Hewgill, B. and McDevitt, H. O., *Mol. Immunol.* 1978. *16:* 51.
20 Quill, H. and Schwartz, B. D., *Mol. Immunol.* 1983. *20:* 1333.
21 Sung, E., Duncan, W. R., Streilein, J. W. and Jones, P. P., *Immunogenetics* 1982. *16:* 425.
22 Rudd, C. E., Bodmer, J. G., Bodmer, W. F. and Crumpton, M. J., *J. Biol. Chem.* 1985. *260:* 1927.
23 Claesson, L. and Peterson, P. A., *Biochemistry* 1983. *22:* 3206.
24 Andersson, L., in Warner, C. M., Rothschild, M. F. and Lamont, S. J. (Eds.) *The molecular biology of the major histocompatibility complex of domestic animal species,* Iowa State University Press, Iowa City 1988, p. 39.
25 Usinger, W. R., Curie-Cohen, M., Benforado, K., Pringnitz, D., Rowe, R., Splitter, G. A. and Stone, W. H., *Immunogenetics* 1981. *14:* 423.

Note added in proof: UR6.4 is now W19 and W8.1 is now W14 [11].
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