THE BOVINE CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX
Serological definition and further characterization of class II haplotypes

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Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
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In this thesis an analysis of the major histocompatibility complex (MHC) class II in cattle is reported, with emphasis on the development of class II serology. First, the production of class II alloantisera, and the serological definition of bovine MHC class II polymorphism is described. Subsequently, the alloantisera were analyzed for the presence of distinct antibody populations specific for the products of the two class II genes DR and DQ, using absorptions, and the MAILA assay. Furthermore, serological defined class II polymorphism was compared with typing data obtained with biochemical techniques (1D-IEF for DRB3, DQA and DQB), and DNA based typing methods (DRB3-PCR RFLP, and DQA and DQB RFLP). The extensive typing for BoLA class II enabled detailed characterization of the class II polymorphism present in Holstein Friesians.
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STELLINGEN

1. Produktie van kwalitatief goede alloantisera voor het typeren van het bovine klasse II polymorfisme is mogelijk door gerichte huidtransplantaties te combineren met absorptie met bloedplaatjes. *Dit proefschrift.*

2. Kruisreactiviteit van anti-HLA klasse II monoklonale antilichamen met de BoLA klasse II moleculen van het rund is niet te voorspellen op basis van de sequentie homologie in het epitoop gebied van de klasse II moleculen van mens en rund. *Dit proefschrift.*

3. Een cel-panel, van uitstekend gekarakteriseerd materiaal en voor iedereen beschikbaar, is onontbeerlijk voor MHC onderzoek. Voor het BoLA onderzoek is het samenstellen van een referentie panel analoog aan dat in het HLA onderzoek derhalve sterk aan te bevelen. *Dit proefschrift.*

4. In het MHC onderzoek bij het rund zijn DRB3-PCR RFLP en microsatelliet typering goed te gebruiken als genetische marker. Echter voor functionele studies betreffende de immuunrespons blijven serologie en 1D-IEF belangrijk. *Dit proefschrift.*

5. De mogelijkheid van een effect van BoLA klasse I compatibiliteit op het optreden van Retentio Secundinarum zoals gesuggereerd door Joosten *et al.* 1991 (*Animal Genetics 22*: 455-463) verdient nadere bestudering, gezien de huidige typeer-mogelijkheden.

6. Typeerreagentia zijn net mensen. Achtergrondinformatie kan aanzienlijk bijdragen tot het begrijpen van hun reaktiepatroon.

7. De kans dat interdisciplinair promotie onderzoek slaagt is zeer klein als de betrokken vakgroepen zich niet mede verantwoordelijk voelen.

8. Indien na het verwerven van het doctoraat een omscholingscursus nodig is om een baan te verkrijgen op de arbeidsmarkt, is er sprake van kapitaalvernietiging.

9. Parasieten brengen vreugde aan, is het niet bij het komen, dan is het wel bij het gaan.

10. De slimsten zijn zij die mensen voor zich laten werken die slimmer zijn dan zij zelf.

11. Op een overbevolkt laboratorium openbaren de dierlijke (territorium)driften van de mens zich op onnavolgbare wijze.

12. Het onderwijs trekt profijt van de snelle ontwikkelingen op het gebied van de automatisering. Dit geldt echter alleen in die landen die er geld voor (over) hebben.

Ph.R. Nilsson
The bovine class II major histocompatibility complex.
Serological definition and further characterization of class II haplotypes.
Wageningen, 21 december 1994

Voor mijn ouders

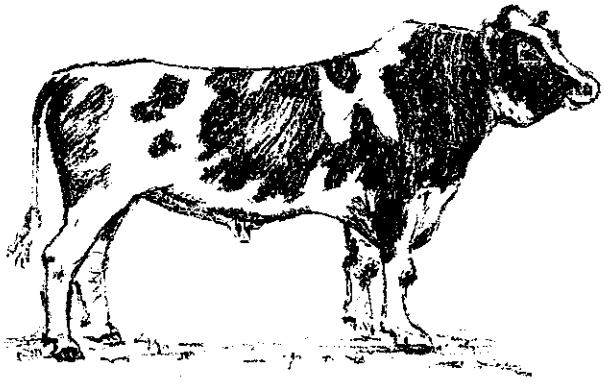
het afgelopen jaar is boek geworden
en trommelt een bekende klank
op mijn beslagen ruit;
de dagen gaan vrij-uit
want ik was mank.

jij en het jaar en ik zijn boek geworden.
[*Neeltje Maria Min*]

CONTENTS

ABBREVIATIONS

AA	amino acids
Ab	antibody
APC	antigen presenting cell
BoLA	bovine lymphocyte antigen
CFDA	carboxyfluoresceine-di-acetate
1D/2D	one/two-dimensional
cM	centimorgans
DH	D-region haplotype
DMSO	dimethyl sulfoxide
DRBF	DRB-region focusing
FCS	fetal calf serum
FIBW	fifth international BoLA workshop
FITC	fluorescein isothiocyanate conjugate
g	gravity
HBBS	hanks' balanced salt solution
HF	Holstein Friesians
HLA	human lymphocyte (leucocyte) Antigen
IEF	isoelectric focusing
Ig	immunoglobulin
kb	kilobase
mAb	monoclonal antibody
MAILA	monoclonal antibody-specific immobilization of lymphocyte antigen
MHC	major histocompatibility complex
Mr	relative mass
NaN_3	natrium azide
NBS	newborn bovine serum
NRS	normal rabbit serum
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCF	two colour fluorescence
RFLP	restriction fragment length polymorphism
RT	room temperature
WAU	Wageningen Agricultural University



Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. Short history of the MHC

The Major Histocompatibility Complex (MHC) is a chromosomal region, where series of extremely polymorphic genes are located. These polymorphic genes encode cell surface proteins that are important in the immune response to different antigens. The first indications for the existence of the MHC were provided by Gorer (1936). He reported on a 'hereditary antigenic difference' in the blood of mice, and this genetically determined bloodgroup antigen appeared to be involved in rejection of transplanted tumors in mice (Gorer 1937). Ten years later, a study on the phenomenon of graft rejection in congenic mouse strains, resulted in the definition of a set of genes coding for the cell surface molecules that determined tissue compatibility (Gorer *et al.* 1948). The molecules involved in the process of tissue rejection were called histo-compatibility (literally tissue compatibility) molecules and the set of genes coding for these molecules in mice was called the H-2 complex (Snell 1948). It appeared that some of the H-2 complex genes were responsible for strong effects on transplantation reactions, whereas others induced only a weak effect. Based upon these observations a distinction was made between genes with 'major' and 'minor' effects. As the genes with 'major' effects appeared to be located on a single chromosome, in a single region, and moreover, as homologous regions were found in other species, the complex was re-defined as the Major Histocompatibility Complex.

Soon after the description of a genetic system involved in graft rejection in the mouse, indications for a similar system in humans were found (Gibson and Medawar 1943). However, in contrast to the fast developments in definition of the mouse MHC, it was not until the beginning of the sixties that extended research on the human transplantation antigens (human leucocyte antigens, HLA) started. From then on, the characterization of the HLA system developed rapidly. With the increasing interest for the HLA, also the research on the MHC of other species developed. At present homologues of the mouse MHC have been detected in some twenty vertebrate species, including cattle (for a review see, Klein 1986).

The first evidence for the presence of MHC in cattle was reported by Amorena and Stone (1978) and Spooner *et al.* (1978). Both studies described independently the

bovine leucocyte antigens (BoLA). Since then rapid progress has been made in characterization of the genetic diversity of the bovine MHC. Today, the MHC of cattle is one of the best characterized MHCs following man and mouse.

2. Structural characteristics of MHC molecules

The MHC molecules are membrane-integrated glycoproteins. They occur at the surface of cells as heterodimers, composed of two non-covalently associated chains. According to differences in structure, cell surface distribution and function, two categories of MHC molecules are distinguished: class I and class II molecules (Figure 1).

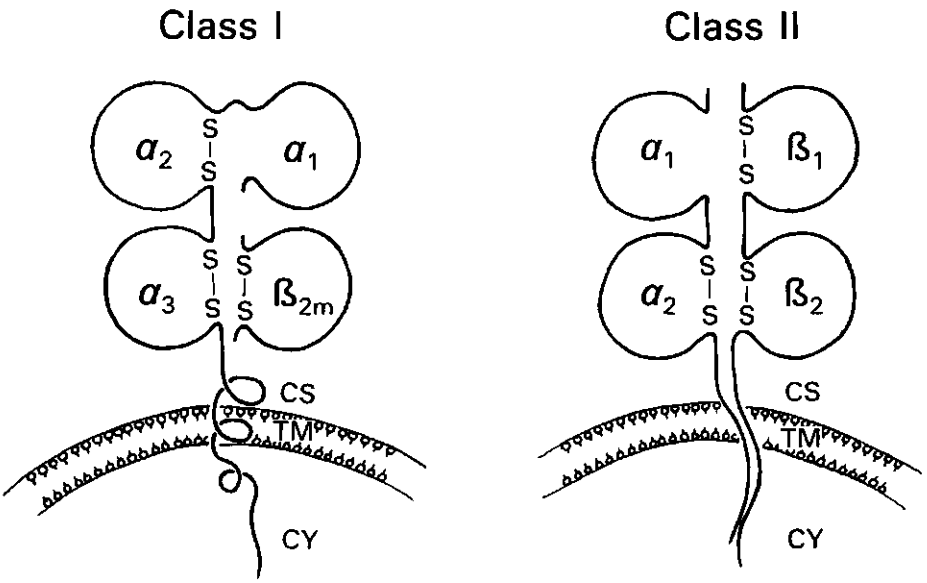


Figure 1. Schematic representation of the structure of class I and class II molecules. Extracellular domains are identified by *α* and ß designations.
ß2m = ß2-microglobulin, TM = transmembrane region, CY = cytoplasmatic region, CS = connecting stalk.

- the class I molecules

Class I molecules are composed of a light chain, $ß_2$-microglobulin ($ß_2$m) and a heavy chain (α) with molecular weights of about 12kD and 44kD respectively. The $ß_2$m chain, is an invariant polypeptide, encoded by a single gene located outside the MHC. The heavy chain is a polymorphic glycosylated polypeptide, and can be subdivided into three regions: an extracellular (external) region, a transmembrane region (TM) and a hydrophobic cytoplasmic tail (CY). The extracellular region is divided into three domains (α1, α2, and α3), and a connecting stalk. Each domain consists of about 90 amino acids. The α1 and α2 domains form the antigen binding. The class I molecules are expressed on all nucleated mammalian cells except brain and germ-line cells (for a review see, Klein 1986).

- the class II molecules

Class II molecules have a domain structure similar to class I. They are composed of an α and a ß chain, both encoded by closely linked MHC genes, and with molecular weights of approximately 35kD and 28kD respectively. Similar to class I α chain, the class II α and ß chains are also polymorphic glycosylated polypeptides, composed of three regions: an extracellular (external) region, a transmembrane region and a hydrophobic cytoplasmic tail. The extracellular region is divided into two domains (α1 - α2, and ß1 - ß2) each of about 90 aminoacids long, and a connecting stalk. The α1 and ß1 domains form the antigen binding site. The class II molecules are mainly expressed on antigen presenting cells (APC) and B-lymphocytes. Expression in many other cell types can be induced by interferon gamma.

- the antigen binding site

Insight in the general structure of MHC molecules, and its antigen binding site was gained not until 1987, when Bjorkman *et al.* (1987a) described the three-dimensional structure of the human class I molecule HLA-A2. It became apparent that the antigen binding site could be described as a groove formed by two α-helices on top of a floor of eight strands of antiparallel ß sheets. The amino acid residues in the ß sheet and α-helices facing the groove are involved in peptide binding (Bjorkman *et al.* 1987b) (Figure 2).

The structure of the human class I molecule (HLA-A2) was subsequently used for the construction of a hypothetical model of the class II binding site, as class I and class II molecules were supposed to exhibit a high degree of similarity in domain structure (Brown *et al.* 1988). Recently, the hypothetical class II model could be

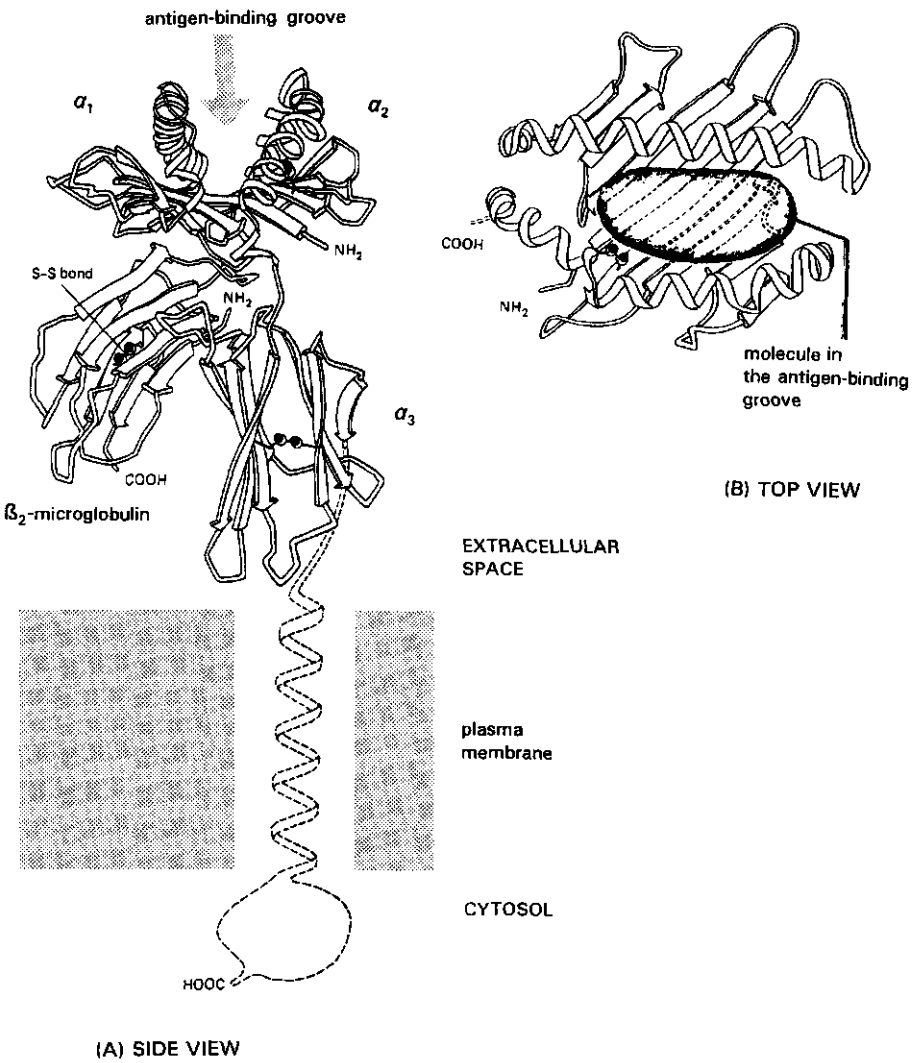


Figure 2. Schematic drawing of the molecular structure of a class I MHC molecule as defined by X-ray crystallography (Bjorkman *et al.* 1987). The α1 and α2 domains constitute the antigen binding site, a groove formed by two α-helices (walls) and a single eight-stranded ß-pleated sheet (bottom) (Modified after Alberts *et al.* 1989).

confirmed (Brown *et al.* 1993). The structure of the class II molecule is similar to that of the class I HLA molecule and to the hypothetical model of the class II binding site based on the class I structure. However, there are fundamental differences between class I and class II molecules which will affect their peptide binding.

The class I peptide-binding site is blocked at both ends. This imposes severe restrictions on the sizes of peptide it can accommodate. Class I proteins generally bind short peptides (8-10 residues), using conserved residues at the ends of the binding site (Falk *et al.* 1991, Jardetzky *et al.* 1991, Hunt *et al.* 1992a). Longer peptides will bulge out in the middle (Guo *et al.* 1992).

The class II binding site is not blocked at either end, which allows peptides to protrude from it. Class II molecules bind longer peptides (15-18 residues), using conserved residues distributed throughout the binding site (Rudensky *et al.* 1992, Hunt *et al.* 1992b, Chicz *et al.* 1992, 1993).

- the genomic arrangement

The structure of the class I and class II molecules is reflection of the genomic arrangement of the coding regions (exons) of the MHC genes. The exons are referred to numerically in the 5' to 3' direction, and separated by introns.

A class I gene consists of eight exons (Figure 3a). The first exon encodes the leader peptide (L). Exons 2 through 4 encode the extracellular region: the α1, α2 and α3 domains. The transmembrane region (TM) is encoded by the fifth exon, and the cytoplasmatic tail (CY) and the 3-untranslated region (3'UT) is encoded by exon six, seven and eight.

For class II, the alpha (α) and beta (ß) chain encoding genes consist of five, respectively six exons (Figure 3b). The first exon encodes the leader peptide, and the second and third exons encode the extracelluar domains of the alpha and beta chains (α1 and α2, ß1 and ß2). For the alpha genes the connecting stalk (CS), the TM, the CY and the 3'UT are encoded by exon 4 and 5. For the beta genes the fourth exon encodes the TM, and exon 5 and 6 the CY, and 3'UT.

3. Function of MHC molecules

Initially the function of the MHC was considered to be limited to the regulation of allograft reactions. However 30 years ago, the true function of the MHC was discovered (Lilley *et al.* 1964). It appeared that MHC molecules are essential in the

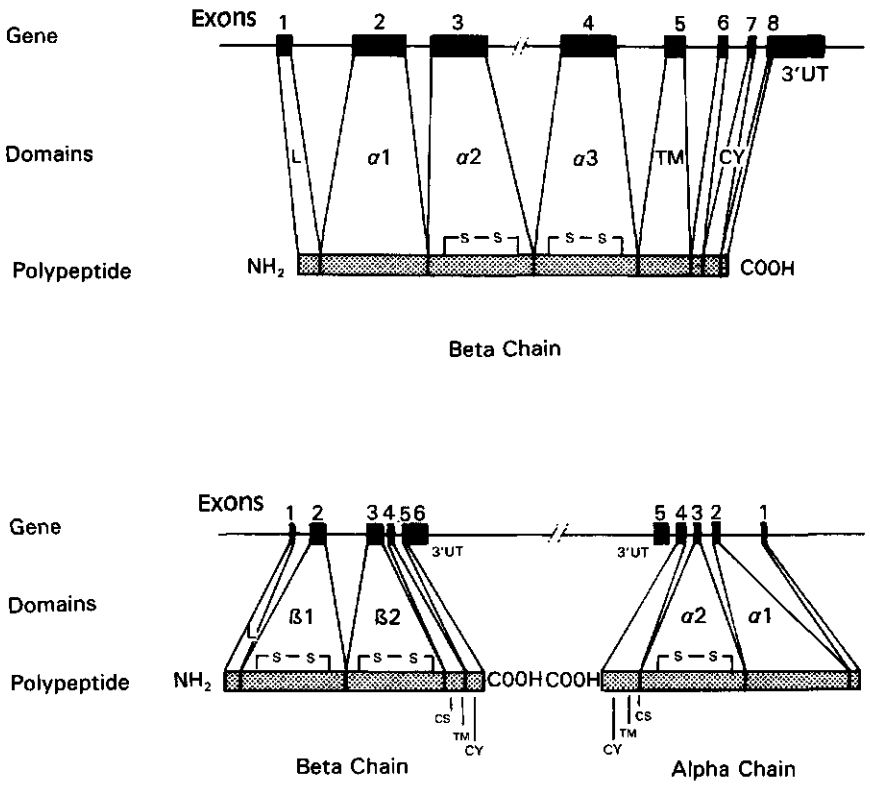


Figure 3a and 3b. Gene and protein structure of class I and class II molecules. A comparison of the exons of a class I gene with domains of a class I heavy chain, and the exons of a class II alpha and beta gene with the respective domains of the class II chain. The genomic DNA that encodes the molecules is shown at the top of the figures, and the different regions of the protein are shown at the bottom. The shown exon-intron structure is based on human genes.
EX = exons, L = leader peptide, TM = transmembrane region, CY = cytoplasmatic tail,
CS = connecting stalk, 3'UT = untranslated regions (Modified after Paul *et al.* 1994).

specific immune response to (foreign) peptides (antigens). In the immune responses three cell types are involved: the antigen presenting cells (APC), the B-lymphocytes (B-cells) and the T-lymphocytes (T-cells). Activation of these cells can lead to two distinct types of immune responses: the humoral (antibody) response and the cell-mediated response.

Activation of the APC by bacteria or adjuvants, takes place during uptake andprocessing of the antigen. B-cells can be induced by direct interaction of antigen with the immunoglobulins on the cell membrane. However, involvement of the T-cells is needed for an optimal response, as the activation of APC and B-cells is regulated, at least in part by cytokines from T-cells.

Before the T-cells influence in the immune response, they must first be activated themselves, which can only be achieved by cell-cell contact with APC. However, the T-cells will not react unless the APC also displays MHC molecules. Only when the antigen is presented in context of 'self' MHC molecules, the T-cell receptor (TCR) can recognize it. This phenomenon, is called MHC-restriction (Zinkernagel and Doherty 1974). The antigens which are presented to T-cells are located in the groove of the MHC molecules (Schwartz 1985, Babbitt *et al.* 1985, Buus *et al.* 1986, Watts *et al.* 1986). In general, one can state that the immune responsiveness of an individual is largely determined by the 'TCR-antigen-MHC' interaction, because (as mentioned above) most B-cells eventually also need help from T-cells for proliferation and antibody production.

Antigen presentation to T-cells can occur in the context of the class I as well as class II molecules. However, there are substantial differences between the two MHC classes, which are summarised in Figure 4. In the endoplasmatic reticulum, the class I molecules bind endogenous peptides derived from endogenous proteins, either self or pathogen (e.g. viruses) derived, and present these at the cell surface. A subpopulation of the T-lymphocytes, the cytotoxic T-lymphocytes (CD8 positive), will recognize the cells that display viral peptides combined with class I molecules, and subsequently mount an immune response by releasing cytokines and cytolysins. This will lead to destruction of the infected APC.

The class II molecules generally present peptides resulting from intracellular processing of endocytosed proteins of extracellular pathogens (e.g. bacteria), or self-proteins. The antigen-class II complex at the cell surface is recognized by helper T-lymphocytes (CD4 positive), which subsequently interact with B-lymphocytes or cytotoxic T-lymphocytes. Studies in man and mice revealed the existence of two subsets of helper T-lymphocytes (TH1 and TH2), each secreting a specific

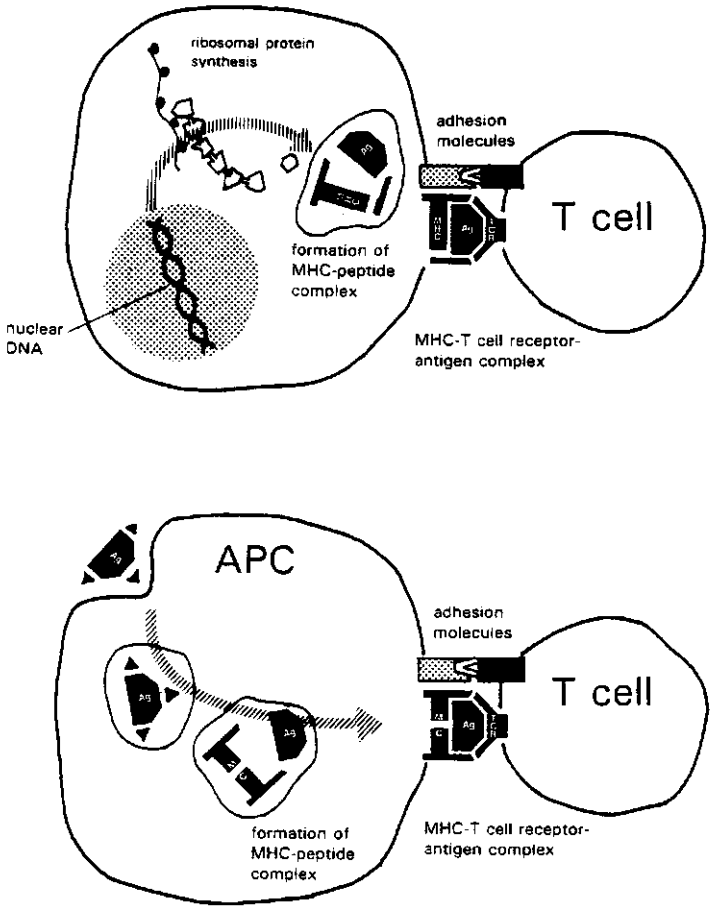


Figure 4. Schematic representation of the interaction between a MHC molecule on a antigen presenting cell (APC), processed antigen (Ag) and a T-cell receptor (TCR). The upper figure represents the formation of a complex of an endogenous antigen (such as viral peptide) with a MHC class I molecule and, the subsequent presentation of this complex to the TCR. The lower figure represents the formation of a complex processed exogenous antigen (such as a bacterial peptide) with a MHC class II molecule, and the subsequent presentation of this complex to the TCR. Exogenous antigen is internalized by APC and processed before binding to the class II MHC molecule (Modified after Joosten 1990).

combination of cytokines, leading to different immune responses (Mosman and Coffman 1989). For example, in mice resistance to the protozoan parasite, *Leishmania infantum*, is preferentially associated with stimulation of TH1 cells, secreting interferon gamma. Interferon gamma has a stimulating effect on IgG_{2a} production by B-cells and macrophages, which appeared to be essential for clearance of the parasite. In contrast, the induction of TH2 cells that specifically secrete interleukin 4 (IL4), is associated with susceptibility to *Leishmania* infection. IL4 suppresses macrophage activation and stimulates the proliferation and differentiation of another subset of B-cells, resulting in the increased production of IgG_1 and IgE (Snapper and Paul 1987). The type of TH effect might be controlled by the MHC class II type (Murray *et al.* 1992).

In cattle, both class I and class II mediated MHC-restriction of T-cell responses have been reported. Bovine MHC class I restriction has been shown in *Theileria annulata* (Preston et *al.* 1983), *Theileria parva* (Goddeeris *et al.* 1990), and bovine herpes virus-1 infection (Splitter *et al.* 1988). Class II restricted variation in T-cell responses has been shown for ovalbumin (Rothel *et al.* 1990, Glass *et al.* 1990), and synthetic Foot-and-Mouth Disease Virus (FMDV) peptides (Glass *et al.* 1991a). The magnitude of the T-cell responses towards the FMDV peptides, and the FMDV peptide-region recognized by the T-cells are influenced by the MHC polymorphism as well (Glass *et al.* 1991b, 1994). Furthermore, MHC class II restriction has been reported for *Oesophagostomum radiatum* antigens (Canals, personal communication).

4. Organisation of the bovine MHC

The bovine MHC is located on the short arm of chromosome 23 (Fries 1986), and its general organisation is very similar to the human MHC. Equivalent to the nomenclature of the MHC genes in man (human leucocyte antigens: HLA), the bovine genes are referred to as BoLA genes.

A tentative map of the bovine MHC is shown in Figure 5. The map has been drawn partly on the basis of the gene order established in man, mouse and rat (Trowsdale *et al.* 1991), as in cattle the exact order of MHC genes has not yet been completely determined. In cattle the MHC can be divided into four regions: class I, class IIa, class IIb and class III.

The class I region is the most telomeric part of the MHC, and the class III (or central) region is located between the class I and the class IIa region. The class IIa

region is tightly linked to the class I region (Usinger *et al.* 1981, Lindberg *et al.* 1988). The distance between the BoLA-DRB3 locus and BoLA-A locus is probably in the same order as the distance found in man between the HLA-DRB region and the HLA-B locus. Based on data from 287 informative meiosis, the recombination distance between the DRB3 locus and the region encoding for both CYP21 (cytochrome P-450 steroid 21-hydroxylase) and BoLA-A, is estimated with 95% certainty on 1 centimorgans (1 cM). No recombinations are found yet between the CYP21 locus and the A-locus (Van Eijk *et al.* 1994).

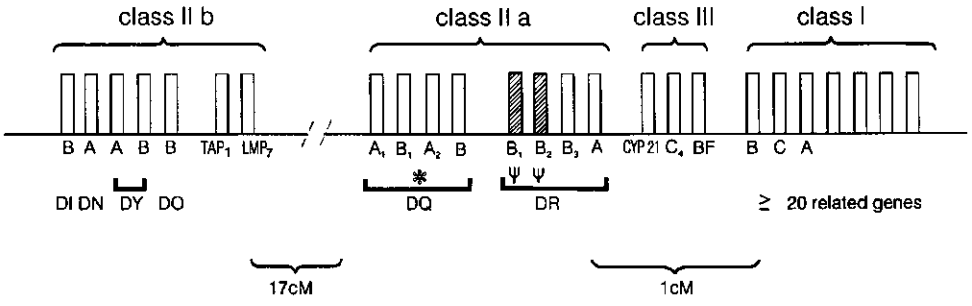


Figure 5. A schematic genetic map of the MHC region on chromosome 23 in cattle. Class I, II and III indicate regions where different groups of MHC encoded genes are located. Individual genes are denoted by boxes.

¥ marks the pseudogenes

* marks the region where the number of genes varies between the haplotypes

-//- indicates a recombination distance of about 17cM

The class IIb region is separated from the class IIa region by a recombination distance of approximately 17 cM (Andersson *et al.* 1988, Georges *et al.* 1990, Van Eijk *et al.* 1993). Andersson *et al.* (1988) suggested that separation of class IIb region from the other three regions was caused by a recombination 'hot spot'. The distance of 17 cM was confirmed by Van Eijk *et al.* (1993) using sperm typing in combination with 'primer extension preamplification'. Such a high recombination distance between class II genes has not been observed in any other mammalian species.

In Figure 5 the class IIb region is located on the 'left' (centromeric) of the class IIa region, but it may also be located to the 'right' (telomeric) of the class I region. The location of this region has not yet been established.

The class I, class III and class IIa loci are generally considered as a genetic unit and are inherited en bloc. A large number of class I and class II allele combinations is found in much greater frequency as one would expect on the basis of the frequencies of each of the involved alleles. This phenomenon is called 'linkage disequilibrium'. The combination of alleles inherited 'en block' is designated as a MHC haplotype.

- the class I region

The class I region is a multigene gene family spanning several hundreds of kilobases (kb) (Bensaid *et al.* 1991), and it encodes the class I molecules. There is some uncertainty on the number of class I loci present in cattle, and little is known concerning the number of class I genes expressed. Investigation of the class I region, performed by Southern blot hybridization utilizing a human class I cDNA probe, indicated the presence of multiple class I genes, possibly 20 or more (Lindberg and Andersson 1988). However, the presence of just two (Ennis *et al.* 1988, Bensaid *et al.* 1991), or even three (Bensaid *et al.* 1991) class I loci has been demonstrated by cDNA cloning experiments. The expression of two class I loci was supported by Ellis *et al.* (1992), based on transmembrane and cytoplasmic domain sequences. The presence of at least three class I loci was supported by Garber *et al.* (1993), based upon comparison of their cDNA sequences with those published previously. Recently Garber *et al.* (1994) isolated from one heterozygous animal six different cDNAs, and indicated again the presence of at least three transcribed class I genes.

Further analysis of the expressed class I genes, showed two highly expressed genes, with the possibility of a third gene with a lower expression level. Surprisingly, there are no locus specific AA in the α1 domain. Possibly, the situation at the genetic level more complex than first thought (Ellis *et al.* 1994).

Expression of more than one class I gene by a single haplotype, has also been indicated by usage of other than the above mentioned DNA based methods. Toye *et al.* (1990) showed differential reactivity of monoclonal antibodies with class I molecules on mouse L cells transfected with DNA from a MHC homozygous cow. Dual expression is further supported by biochemical studies on the class I molecules (Joosten *et al.* 1992).

Based on all the above mentioned studies, using biochemical and molecular techniques, expression of two different class I molecules per haplotype is now generally accepted. One of the two class I molecules is supposed to be encoded by the BoLA-A. The other represents the products of a second locus, which may be the 'B' locus, or the 'C' locus (Ellis *et al.* 1992).

In contrast to the above mentioned evidence of two expressed loci, there is scant evidence for more than one single segregant serological series, which implies that the two expressed class I loci are very tightly linked and make up a conserved block. This is supported by the fact that the two class I loci described by Bensaid *et al.* are less than 210 kB apart (Bensaid et *al.* 1991). As the sera define a single segregant series of alleles, the serotyping is considered as A-locus specific. However, strictly speaking, class I polymorphism defined by class I sera, has to be considered as class I haplotype polymorphism.

- the class IIa and IIb regions

The existence of one DRA locus and three DRB loci, two DQA and two DQB loci, one DOB, one DYA, one DYB, one DIB, and one DNA locus have been described in cattle (Andersson *et al.* 1986a, 1986b, 1988, Andersson and Rask 1988, Muggli-Cockett and Stone 1988, 1989, Groenen et *al.* 1989, Stone and Muggli-Cockett 1990). These class II genes are located in one of the two class II subregions, class IIa or class IIb.

The class IIa region contains the DRA, DRB, DQA and DQB loci and encodes for class II DR and DQ molecules. Investigation by Southern blot analysis using human probes indicated the presence of a single DRA and at least three DRB loci (Andersson and Rask 1988). For the DRA gene a fairly low level of polymorphism was found (Andersson et *al.* 1986b, Sigurdardóttir *et al.* 1988). Isolation of genomic clones of three DRB genes (DRB1, DRB2 and DRB3) and comparison with the human DRB genes, showed that exon 2 (encoding the ß1 domain) of the DRB3 gene is conserved, whereas the DRB1 and DRB2 genes are quite divergent (Groenen *et al.* 1990, Sigurdardóttir *et al.* 1991a). To date, it is generally accepted that only DRB3 is a functional gene, whereas DRB1 and DRB2 are regarded as pseudogenes.

Studies of bovine MHC class II genes at genomic level revealed considerable DQ polymorphism. Two distinct DQA genes were reported, represented by the genomic clones W1 (Van der Poel *et al.* 1991) and A5 (Sigurdardóttir *et al.* 1991b), respectively. Also polymorphism in the copy number of DQB genes has been

reported, four DQB genes were reported: DQB1, DQB2, DQB3 and DQB4. Furthermore, the number of DQA and DQB loci present in one haplotype appeared to vary between individuals. Three loci combinations are described: a single DQA (W1) and a single DQB locus (DQB1), two DQA loci (A5 + A5) and a single DQB locus (DQB1 or DQBx), and two DQA loci (W1 + A5, or A5 + A5) and two DQB loci (DQB1 and DQB2, DQB4 or DQBx). This variation in number of DQA and DQB loci has been confirmed by PCR amplification and cloning experiments (Sigurdardóttir *et al.* 1992, Van der Poel, personal communication). In a recent study, for three haplotypes carrying DQB duplications, evidence has been presented for the expression of both DQB genes (Xu *et al.* 1994). However, it is not clear, neither for DQA, nor for all the other DQA and DQB gene combinations, to what extent the DQ polymorphism is expressed at the cell surface.

As mentioned above, the class IIb region is separated from the rest of the MHC by a recombination frequency of approximately 17%. Southern blot analysis revealed the presence of the DOB, DNA, DYA, DYB, and DIB genes (Andersson *et al.* 1988, Andersson and Rask 1988). These genes do not appear to be highly polymorphic, and there is no evidence for their expression at the cell surface.

In man, several new genes such as peptide transporter genes (Transporter associated with Antigen Processing, TAP) and proteasome-related genes (Large Multifunctional Protease, LMP) are recently described to be located within the MHC region. Recently bovine homologues of these 'new genes' have been detected, TAP1 and LMP7 (Davies *et al.* 1992), which might be located in the bovine IIb region (Davies, personal communication).

- the class III region

So far only limited efforts have been made to characterize the bovine class III or central region. In man this region contains numerous genes, such as genes for the 21 hydroxylase (CYP21), complement 4 factor (C4), complement factor B (Bf), tumor necrosis factors A and B (TNFA and TNFB), heat shock protein 70 (HSP70) and a number of genes with unknown functions (Trowsdale *et al.* 1991). In cattle, only the genes coding for CYP21, C4, and Bf have been mapped. This was done by linkage analysis (Andersson *et al.* 1988, Teutsch *et al.* 1989, 1990), or by analysis of somatic cell hybrids (Skow *et al.* 1988).

The class III region genes are neither structurally nor functionally related to class I or class II genes, and it has been suggested (Klein 1986), that the class III region is not part of the MHC, but resides on the same chromosome by coincidence.

Dawkins *et al.* (1987), however, hypothesised that the organization of the complete MHC (class I, II and III) has been preserved, and if the complete MHC existed before mammalian speciation, this preservation might have been maintained during the evolution for more than 75 million years. This hypothesis was based upon the observation of the occurrence of fixed combinations of alleles in the class II and/or class I region and the class III region, that maintained in a stable state throughout many generations. This phenomenon is referred to as supratypes (Dawkins *et al.* 1983), preferential allelic association (Festenstein *et al.* 1986), or extended haplotypes (Awdeh *et al.* 1983), and it is suggested that these fixed combinations of alleles can be used to mark extensive segments of the MHC, assuming that the segments are more important than the individual alleles.

The reason for the occurrence of this phenomenon is yet unknown, but involvement in immune regulation, by some of the class III genes, such as macrophage regulatory factors that affect B cell proliferation and antibody production (French and Dawkins 1990), might be in accordance with this hypothesis.

5. Detection of bovine MHC polymorphism

Several methods are available for detection of MHC polymorphism in cattle. As the MHC genes are expressed co-dominantly, the products of both genes (paternal and maternal) of an individual can be detected on the cell surface.
Class I definition is mainly performed by serotyping (Bull *et al.* 1989, Bernoco *et al.* 1991, Davies *et al.* 1994a). Immunoprecipitation and subsequent one-dimensional isoelectric focusing (1D-IEF) is also used (Joosten *et al.* 1988, Lindberg and Andersson 1988, Oliver *et al* 1989, Watkins *et al.* 1989a), although to a very low extent. Class I DNA typing is not operational.

Several methods have been used for class II typing. Its first definition was on the basis of mixed lymphocyte culture (MLC) typing (Usinger *et al.* 1977, 1981, Curie-Cohen *et al.* 1978, Emery and McCullagh 1980, Splitter *et al.* 1981, Newman *et al.* 1982a, Davies and Antczak 1991a). Next to MLC typing, also typing with T-cell lines and clones have been performed (Teale and Kemp 1987). At this moment however, for typing of expressed products, immunoprecipitation combined with 1D-IEF

(Watkins *et al.* 1989b, Joosten *et al.* 1989, 1990, Bissumbar *et al.* 1994), and serotyping are used (Newman *et al.* 1982b, Nilsson *et al.* 1994). For class II typing at the genomic level, at first instance only restriction fragment length polymorphism (RFLP) was been used (Sigurdardóttir, 1988, 1991b). Recently, for DRB3 typing, two other DNA based methods for class II typing have been developed: DRB3 PCR-RFLP typing (Van Eijk *et al.* 1992) and DRB3 microsatellite PCR (Ellegren *et al.* 1993). Below the principles of the four typing methods used in this thesis are given.

Serology

Serotyping is based on recognition of the MHC molecules by antibodies. The method used is as follows: peripheral blood mononuclear cells (PBMC), freshly isolated, are suspended in a serum fraction. Subsequently, complement is added. If serum-antibodies recognize MHC molecules on the cells, cell-lysis will occur, which can be visualised using a fluorescent dye. After screening the reactivity of a large number of sera, the MHC type is deduced from the pattern of non-reactivity versus reactivity.

In cattle, serotyping is performed with alloantisera, which are polyspecific. BoLA specific monoclonal antibodies (mAb) are not yet available for typing purposes. Class I alloantisera have been obtained from parous cows (Iha *et al.* 1973, Caldwell *et al.* 1977, Spooner *et al.* 1979, Newman and Hines 1979, 1980, Amorena and Stone 1980, 1982, Dufty and Outteridge 1985), since a maternal immune response to paternally inherited fetal MHC antigens can occur during pregnancy (Redman *et al.* 1987). Another method used is planned immunization. Immunizations for production of class I sera have been performed through injection of leucocytes (Caldwell *et al.* 1977, Spooner *et al.* 1978, Amorena and Stone 1980, 1982), or by subcutaneous skin implantation (Spooner *et al.* 1978, Pringnitz *et al.* 1982, Amorena and Stone 1982). Alloantisera obtained by planned immunization usually give higher antibody titers than the sera obtained from parous cows.

For several reasons, production of bovine class II typing sera has proven to be more difficult than the production of class I typing sera. As in man, bovine class II antigens are also expressed on B-lymphocytes and not on resting T-lymphocytes (Spooner *et al.* 1984). B-lymphocytes mount only circa 33% of the total lymphocyte population (Lewin *et al.* 1985). Moreover, B-lymphocytes are also positive for class I antigens, which means that class II typing sera should not contain class I directed antibodies.

Class II typing sera without class I reactivity, can be obtained in two ways. Immunizations between 'class I identical class II non-identical' animals (Arriens *et al.* 1991, Williams *et al.* 1991, Davies and Antczak 1991b), or immunizations between 'class I and class II non-identical' animals with subsequent platelet absorption of the sera to remove the anti class I reactivity (Cwik *et al.* 1979, Newman *et al.* 1982b, Mackie and Stear 1990, 1992, Nilsson *et al.* 1994).

Apart from this, an overall lack of knowledge of the bovine class II haplotypes in the nineteen-eighties may have hampered the production of class II sera to some degree. Furthermore, the apparent complexity of the class II region has frustrated the interpretations of class II antibody reactivity.

Immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF)

This procedure is based on size and charge differences of MHC molecules. Molecules are metabolically labelled with ^{35}S. After lysis of the cells, the MHC molecules are precipitated with class I or II specific mAb. To detect the variance of the precipitated molecules, separation by 1D-IEF is performed. With some exception, the MHC molecules are expected to differ in isoelectric points (due to differences in amino acid composition), and therefore the molecules will focus into different regions of the gel. The banding patterns evolved by the 1D-IEF separation are visualised on autoradiograms, and the MHC type is deduced from these patterns. In cattle 1D-IEF has proven to be very useful for class II analysis (Joosten *et al.* 1990). However, 1D-IEF is a time consuming and costly technique.

Restriction fragment length polymorphism (RFLP)

For RFLP typing, isolated and purified genomic DNA is digested with the restriction enzymes *Pvu*II or *Taq*I. The restriction fragments are separated based upon size by agarose gel electrophoresis, and transferred to a nitrocellulose filter. Subsequently, a radioactive labeled probe (^{32}P), recognizing a fragment of the gene under study, is hybridized to complementary DNA on the filter. The fragments containing the gene under study will be of different size due to polymorphism in the restriction sites, and different banding patterns will evolve. These banding patterns are visualised on autoradiograms and the MHC type is deduced from these patterns.

A negative aspect of RFLP typing is the overestimation of polymorphism, because the restriction sites are also present in introns, and false positive hybridisation of

probes with other DNA fragments may occur. Furthermore, RFLP typing is very laborious, and the patterns are not always easy to interpret. For molecular bovine DQ typing however, no other DNA based method is yet available.

Polymerase chain reaction-RFLP (PCR-RFLP)

Genomic DNA is used for amplification of the DRB3 alleles by PCR. The PCR products are subsequently digested with three restriction enzymes, *Rsa*I, *Bst*YI and *Hae*III. The restriction fragments are different in size due to nucleotide differences associated with the different alleles. Fragments separated by polyacrylamide gel electrophoresis thus will produce different banding patterns. These banding patterns are visualised by ethidium bromide staining, and the DRB3 type is deduced from these patterns.

PCR-DRB3 RFLP is a rapid and sensitive method for the detection of DRB3 exon 2 polymorphism. However, it has its limitations. It is not always possible to define an individual genotype, as there are at least twelve pairs of DRB3.2 genotypes that can not be distinguished by PCR-RFLP eg. DRB3.2*08/DRB3.2*28 versus DRB3.2*10/DRB3.2*26 (Van Eijk, personal communication). Therefore, application of this method should be combined with an other DRB3 typing method, e.g. DRB3 microsatellite PCR typing (Ellegren *et al.* 1993). Within a sire line or family, class I typing could also be used as a marker for the DRB3 type.

6. Bovine class I and class II polymorphism

Since the first report on bovine MHC, five international bovine lymphocyte antigen workshops have been conducted. These workshops are aimed at the characterization of the multiple MHC genes, their polymorphism and the unification of their nomenclature. In the first three workshops only the serum-defined class I specificities were investigated. In the last workshop, Interlaken 1992, also serum-defined class II specificities were analyzed. Moreover, at that occasion also the biochemical typing for class I and class II polymorphism was presented as well as several newly developed DNA based molecular typing methods for class II polymorphism.

- *class I*

Class I polymorphism has been defined most extensively by serology. To date 53 class I specificities are defined: 27 BoLA-A locus (A) and 26 provisional workshop (w) specificities (Table 1). Table 2 gives an overview of the broad class I specificities and their splits. Isoelectric focusing detected the same splits as the sera. Moreover, this technique also detected heterogeneity in three serological specificities (A15(A8),

Table 1. Class I specificities defined in the Fifth International BoLA Workshop (1992) (Davies *et al.* 1994b).

BoLA class I specificity @	Old or local specificity names	BoLA class I specificity	Old or local specificity names
w1		w28	
A2	w2	w29(w28)	w28.1
A3	w3	A30	
w4(w50)	w4	A31(A30)	w12.2
A5	w5	A32	w32
A6	w6	w33	
A7(w50)	w7	A34	
A8	w8	A35(A5)	
A9	w9	w36(A20)	
A10(w50)	w10	w37(A20)	
A11	w11	w38(A6)	
A12(A30)	w12, w12.1	w39(A7)	
A13	w13	w40(A14)	
A14(A8)	w14, w8.1	w41(w49)	
A15(A8)	w15, w8.2	w42	
A16	w16	w43	
w17(A6)	w6.1	w44	
A18(A6)	w18, w6.2	w45	
A19(A6)	w19, w6.4	w46	
A20	w20	w47	
A21	w21	w48	
A22(w49)	w22	w49	
A23(A5)	w23, w5.1	w50	
A24	w24	w51(w28)	COD-cph22
w25		w52	BER-Be2, COD-cph43
A26	w26	w53(w28)	COD-cph63
w27(A10)	w10.1		

@ Supertypes shown in parentheses.

Table 2. Broad class I specificities and their splits in cattle.

Broad	Splits
A5	A23, A35
A6	w17, A18, A19, w38
A7	w39
A8	A14, A15
A10	w27
A14	w40
A20	w36, w37
w28	w29, w51, w53
A30	A12, A31
w49	A22, w41
w50	w4, A7, A10

A22(w49) and A18(A6)) (Davies *et al.* 1994a). As yet DNA based typing is not performed for class I. However, DNA sequencing has been performed. To date nine class I $\alpha 1$ and $\alpha 2$ domain DNA sequences have been published (Garber *et al.* 1993).

- *class IIa (DR-DQ)*

The polymorphism of DR and DQ genes has been defined most extensively by restriction fragment length polymorphism (RFLP). In the Fifth International Workshop the two new molecular techniques: DRB3 PCR-RFLP (Van Eijk *et al.* 1992) and DRB3 microsatellite PCR (Ellegren *et al.* 1993) have contributed considerably to the class IIa definition. Biochemical typing and serology also contributed to the definition of class II at the product level. Thirty-eight class IIa (DR-DQ) and 5 class IIb (DYA-DOB-DIB) haplotypes were defined. A summary of the IIa haplotypes is given in Table 3. To date 36 DRB3 and 19 DQB DNA sequences are defined (Sigurdardóttir *et al.* 1991a, Andersson *et al.* 1991, Ammer *et al.* 1992, Stone and Muggli-Cocket 1992, Xu *et al.* 1993, 1994).

7. Genetic terminology

As mentioned above, the combination of the different MHC alleles of an individual present on one chromosome is designated as a MHC haplotype. The MHC genotype

Table 3. Class IIa haplotypes (DH) defined in the Fifth International BoLA Workshop (1992), sorted by DRB3 PCR-RFLP (Davies *et al.* 1994).

DH	Class II serology	DRB @ 1D-IEF	DQA RFLP	DQB RFLP	DRB3 PCR-RFLP
01A	ND	9	5	5	1
03A	Dw1; Dc6	5	10	10	3
03B	Dc6	12	7F	7E	3
07A	Dw2	2	2	2	7
08A	Dw1; Dc12	6	12	12	8
09A	Dc13	U19	3A	3B	9
09B	ND	ND	3B	3B	9
10A	ND	7	14	14	10
10B	ND	6	14	14	10
10C	Dw1,8	6	11B	11A	10
11A	Dc13,20	1	3A	3A	11
11B	Dc13,20	1	3B	3B	11
11C	Dw1	6	9A	9A	11
12A	Dc5	6	13B	13B	12
12B	Dc5	6	7C	7E	12
15A	Dw3	U17	1D	1	15
15B	Dw3	6	1E	1	15
15C	ND	U18	3B	3B	15
16A	Dw1,8	5	11A	11C	16
17A	Dw1	ND	11D	11E	17
18A	Dw1,4; Dc7	4	5	5	18
20A	Dw3	U21	1D	1	20
21A	ND	4	8	8	21
22A	Dc13	11	3C	3C	22
22B	Dw1; Dc9,12	7	9B	9B	22
22C	Dw1,8; Dc9	7	11A	11A	22
22E	Dw1; Dc9	7	11F	11C	22
22F	Dc9	7	3A	4	22
22G	ND	11	3B	3C	22
23A	Dw3	10	7D	7A	23
24A	Dw3	3	1A	1	24
27A	Dw3; Dc11	7	1B	1	27
28A	Dw3	8	7A	7A	28
31A	Dw1,4	2	5	5	31

DH Class IIa haplotype reference numbers
@ Numbers preceded by U are UDF types (= only defined by Joosten, Utrecht).
ND Not determined.

of an individual is made up of two MHC haplotypes: each individual receives one paternal and one maternal haplotype.

Due to the extensive polymorphism, most animals are heterozygous for each allele of a particular MHC gene. However, sometimes an allele or even an allelic combination is present at a relative high frequency in a given population, and some animals can become homozygous for that particular allele, or that specific allelic combination. Homozygosity for a certain allele of one gene, does not automatically imply haplotype homozygosity, as it only refers to the gene studied. In cattle, subsequent insemination of one cow by the same sire seldom occurs. Most families are half sib families, which diminishes the chance of observing two sibs that are genotypically identical by descent.

8. Relevance of MHC typing

The biological significance of the MHC molecules lies in providing context for the recognition of potentially immunogenic peptides, a crucial step in tolerance induction or the initiation of an immune reaction, but other functions can not be excluded. The capacity of a given MHC molecule to bind a peptide and to form a complex is determined by its polymorphism (i.e. structural variance). In other words, the MHC alleles carried by an individual will influence whether the individual mounts an appropriate or inappropriate immune response to a given antigen, be it self or non-self derived. However, a particular MHC allele (haplotype) might confer susceptibility or resistance to a certain infectious disease, but may be protective against other agents. MHC polymorphism is an adequate answer of a population to the problems caused by many diseases: some individuals will be sacrificed because they succumb to certain agents, but the majority will survive and develop immunity.

The relevance of MHC polymorphism for the regulation of the immune response, stimulated many researchers to study association between disease and the MHC. In man, numerous diseases (autoimmune, immune complex-mediated and non-immune) are shown to be associated with particular HLA alleles or even haplotypes (Todd *et al.* 1987). Some of these HLA-associated diseases are: ankylosing spondilytis, coeliac disease, juvenile diabetes mellitus, graves disease, systemic lupus erythematosus, myasthenia gravis, narcolepsy, nephrotic syndrome, psoriasis, rheumatoid arthritis. Also susceptibility to typhoid, yellow fever and malaria is HLA associated (Piazza *et al.* 1972, De Vries *et al.* 1976, 1979, 1989, Hill *et al.* 1991).

The numerous findings in HLA associations, subsequently stimulated many scientists to look for such MHC associations in cattle, because these data could have practical applications and could also be of great economical value. The first report on MHC-disease associations in cattle came from Solbu *et al.* (1982), who analyzed the effect of BoLA class I on mastitis among cows of Norwegian Red breed. These data were later confirmed by Larsen et *al.* (1985), Solbu and Lie (1990), and Våge *et al.* (1992). A number of other disease association studies were performed in cattle, next to association studies on mastitis (Oddgeirssen *et al.* 1988, Weigel *et al.* 1990), these include, bovine virus diarrhoea (Dam and Østergård 1985), bovine leukaemia virus infection (Lewin and Bernoco 1986, Lewin *et al.* 1988), tick and worm infestations (Stear *et al.* 1984, 1988, 1990), ocular squamous cell carcinoma (Stear *et al.* 1989a) and ketosis (Mejdell *et al.* 1994). Most of these investigations in cattle have been reviewed by Østergård *et al.* (1989). They concluded that the statistical models used for analysing the MHC-trait associations in these studies were descriptive rather than analytical, and suggested that more elaborate statistical methods should be applied in the future.

To date virtually all disease association studies in cattle have used class I polymorphism as marker for the entire MHC haplotype. In only three studies disease association with class II polymorphism have been analyzed (Lundén *et al.* 1990, Van Eijk *et al.* 1992, Xu *et al.* 1993). This is mainly due to the shortcoming of the techniques available for an accurate way of defining bovine class II polymorphism in large groups of animals. This is reminiscent of the situation in man some 15 years ago. Originally only class I typing could be achieved and many of the diseases appeared to be associated with HLA class I alleles. Soon after characterization of the class II region became reality, the number of publications on and significance of MHC class II associated diseases increased, particularly for rheumatoid arthritis, juvenile diabetes mellitus and coeliac disease.

In addition to MHC-disease associations, MHC effects on other traits like ovulation rate, body weight and milk production have been proposed (Hines *et al.* 1986, Batra *et al.* 1989, Stear *et al.* 1989b, 1989c, Beever *et al.* 1990, Weigel *et al.* 1990, 1991). Unfortunately, these studies indicated only weak associations which were difficult to confirm.

In connection to the relevance of the MHC polymorphism in the immune response, MHC polymorphism may also be involved in reproduction. In man, during normal pregnancy, a maternal immune response to paternally inherited fetal MHC antigens occurs, which is elicited by MHC class I and to a lesser extent, class II

antigens (Redman *et al.* 1987). The function of this immune response, in relation to a successful pregnancy is not clear, but it seems to be neither harmful nor directly beneficial to a successful pregnancy. Several hypotheses have been proposed. The immune response may result in the production of specific interleukins, that are required for normal placental maturation (Wegman *et al.* 1988), whereas lack of allorecognition (due to maternal-fetal compatibility) would results in abnormal placental development and retention of the placenta (Gill *et al.* 1983). Recently, it has been suggested that MHC compatibility can have a negative effect on the success of *in vitro* fertilization and tubal embryo transfer (Ho *et al.*, cited by Gill 1993). In cattle, also a maternal immune response to paternally inherited fetal MHC antigens occurs (Newman *et al.* 1979, 1980): the first sources of class I typing sera in cattle were parous cows. Additional, evidence for involvement of bovine MHC compatibility in reproduction was presented by Joosten *et al.* (1991a, 1991b). They hypothesized that MHC incompatibility between dam and calf would facilitate the expulsion of the placenta. In accordance to this class I compatibility would be associated with retention of the placenta (for review, see Joosten *et al.* 1992).

Finally, there is one other interesting aspect on MHC worth mentioning, which is its role in kin recognition, in the context of mating. Recent work, using laboratory rodents, established quite convincingly, that the MHC has fundamental effects on the production of individual odours, that can be used in discrimination between individuals. Based on these observations, it has been suggested that preference in mating is controlled genetically, and that the involvement of MHC through the sexual process is, to enrich the genetic resources of offspring, in pursuit of genetic dissimilarity (for review see, Brown and Eklund, 1994). In cattle, where artificial insemination is usually performed, this aspect of the MHC, is of course, not of direct relevance. However, under natural conditions it may be.

9. Aims and outline of the thesis

The important role of the MHC molecules in the regulation of the immune response, the numerous associations of MHC alleles with disease susceptibility in man, and above all the wish to improve disease resistance in cattle based on the genetic evaluation of immune responsiveness and subsequent selection, can be considered as the major impetus to study the bovine MHC.

In 1988, the knowledge of the genetic organisation and polymorphism of the bovine MHC region, especially of the class II region, was very limited. It was hypothesized, that soon after the improvement of the characterization of the class II region, the identification and significance of class II associated diseases in cattle would increase dramatically. In accordance with this quest, the present study started with analysis of class II polymorphism in cattle, with a strong emphasis on the class II serology. Serology is informative on expressed MHC polymorphism, and can be used as a relatively simple tool in disease association studies.

BoLA class II serology was still in its infancy at the time this study started. Several techniques routinely used in human serotyping had to be modified, and class II sera had to be produced. Worldwide, there was very little experience in production of bovine class II alloantisera. We decided to produce alloantisera by skin implantation between class I and class II incompatible donor-recipient pairs (e.g. mother-daughter and half-sib combinations), and subsequent absorption with platelets (to remove the class I reactivity). In Chapter 2, an overview of all immunisations performed for this study is presented, as well as the outcome of these immunisations. Furthermore, in Chapter 2, relevant technical aspects of methods used in this thesis are presented.

The produced B-lymphocyte reactive alloantisera were analyzed together with alloantisera produced by three other research groups working on class II serology (Chapter 3). This analysis demonstrated the presence of 18 clusters, which were closely associated. There appeared to be two main clusters, defined as class II specificities Ds01 and Ds03, with which eleven other clusters were closely associated. The bovine class II alloantisera supposedly contain a mixture of antibodies specific for products of both DR and DQ loci. Discrimination between antibodies specific for DR or DQ is difficult due to linkage between DR and DQ loci. To investigate this aspect in more detail, the Ds03 related alloantisera were analysed by cross-absorption and by application of the monoclonal antibody-specific immobilization of lymphocyte antigen assay (MAILA). The MAILA was modified to enable utilization in cattle (Chapter 4).

A second method which had to be modified extensively was the 1D-IEF method, used for detection of expressed bovine DRB3 polymorphism. This method was adapted for biochemical characterization of bovine MHC DQ allelic variants, because biochemical data on DQ, in addition to those on DRB3, were considered as indispensable in further analysis of the alloantisera for DR and/or DQ specificity (Chapter 5).

In the time the class II serology was developed, great progress was made in the analysis of the class II region, and DNA based methods applicable for class II typing of large numbers of animals became available. This development decreased the necessity for class II serology in population studies, but on the other hand enabled comparison of the developed class II serology with polymorphism detected at DNA level. In Chapter 6, data on DNA based typing of class II MHC are presented (RFLP of DQA and DQB and DRB3-PCR RFLP). The comparison of class II polymorphism as defined by the DNA typing methods with the results of the typing of expressed polymorphism (serology and 1D-IEF) was informative and enabled definition of bovine MHC haplotypes. As information on the occurrence of the polymorphic characters in the different breeds is limited to frequency distribution of class I, the frequency distribution of these MHC haplotypes as present in the typed animals was presented (Chapter 6).

The knowledge about the bovine class II MHC as defined in Chapter 6 was combined with all available exon 2 AA sequences of DRB3 and DQB. It was hypothesized that conserved polymorphic sequences might be selectively recognised by mAb directed against HLA class II sequences. Reactions of such mAb are presented in Chapter 7.

Finally, Chapter 8 provides a general discussion on the data presented in this thesis, and the main conclusions drawn from these observations.

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Chapter 2

TECHNICAL ASPECTS

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To define MHC polymorphism a large number of techniques and methods are available, which are all (in general), published in detail. A selection of these methods have been used in this thesis, however in most cases slight modifications were introduced. Therefore in this chapter the technical aspects of the methods that are relevant for this thesis are presented.

1. Serum production

Immunization (Pringnitz *et al.* 1982)

Immunization for class II alloantisera was performed by skin implantation between animals differing in class I and class II specificities. Forty nine immunizations have been performed, spread over three groups of animals (Table 1). In the first group (A1102 - A1983), donor - recipient pair selection was only based on class I serology. In the second (O0454 - O0557) donor - recipient pair selection was based on class I serotyping, and class II serotyping using a limited set of class II sera, and biochemical typing (1D-IEF) for DRB3. In the third group (A2191 - A2301), selection was not only based on class I and class II serotyping, but also on biochemical typing (1D-IEF) for DRB3. Skin implantation was performed once, twice or three time, as indicated in Table 1. Serum was collected prior to immunization and 18-21 days after the last immunization.

Serum absorption

To remove class I specific antibodies serum absorption was performed, using platelets or lymphocytes.

- platelet isolation

The platelets were separated by differential centrifugation of freshly collected blood, using 15% EDTA (10:1 v/v) as anti-coagulate. Blood was diluted with an equal volume of Phosphate Buffered Saline (PBS)/1%EDTA pH=7.4, and centrifuged (10 min, 600 g, RT, no brake). The supernatant (the platelet rich plasma) was collected carefully, and the procedure was repeated once more. The platelets were pelleted by

centrifugation of the platelet rich plasma (20 min, 2800 g, RT), resuspended in a small volume of PBS/0.1% NaN_3. To remove erythrocytes in the platelet suspension lysis was performed: the platelet suspension was diluted with an equal volume of lysis buffer (0.826% NH_4Cl/0.1% $NaHCO_3$/0.0037% EDTA pH7.4), put 10 min on ice, and then washed with PBS/0.1% NaN_3. The platelets were stored at 4°C at least 1 month and then washed three times with PBS/0.1% NaN_3 before absorption. The average yield was: 1.5 ml platelet pellet per 500 ml of whole (EDTA) blood.

- platelet absorption

The platelets were isolated from the tissue donor in the alloimmunization, or pooled from several animals each carrying a class I antigen specificity that had to be removed from the sera. Equal volumes of serum and platelet of packed platelets were carefully mixed and subsequently incubated for 45 min at RT, and 45 min at 4°C. During incubation the platelets were gently shaken. Thereafter, the platelets were removed by three subsequent centrifugations (15 min, 17440 g, 4°C), and the sera were stored at -80°C. The absorption procedure was repeated once if necessary.

- lymphocyte isolation

Peripheral blood collected in lithium heparin (14 I.E/ml) was centrifuged (10 min, 1000 g (2250 rpm), RT). The 'buffy-coat' was aspirated and diluted with an equal volume of phosphate buffered saline (PBS). This suspension was carefully layered upon Ficoll-metrizoate (sg 1.077, Lymphoprep, Nycomed, Pharma AS). The tubes were spun (20 min, 1000 g), and the peripheral blood mononuclear cells (PBMC) were harvested from the interface and washed twice with Hanks Balanced Salts (HBBS, w/ 0,375% $NaHCO_3$, Gibco LTD), with 0,4 ml of Heparine (5000U/ml) per liter of HBBS. Finally the cells were resuspended in RPMI-1640 (Dutch modification, w/ 20mM Hepes, w/ L-Glutamine, w/ 0.1% $NaHCO_3$, Imperial Laboratories).

An alternative (quick) method used for lymphocyte isolation was performed when from a large number of animals, a small amount of cells had to be prepared. Peripheral blood collected in lithium heparin (14 I.E/ml) was diluted with an equal volume of PBS, and 1 ml was layered upon 0.75 ml Ficoll-metrizoate in a 2 ml

Table 1. The MHC genotypes of recipient and donor animals involved in the production of class I and/or class II typing sera. For class I the serotype (A,w,P) is given, for class II the DH-code, which is based on class II serology, 1D-IEF for DRB3, RFLP for DQA and DQB, and RFLP-PCR for DRB3. ☞

RECIPIENT ID	CII-CIII	/	CII-CIII	DONOR ID	CII-CIII	/	CII-CIII	Number of immuniz.
A1102	w38-03A	/	A16-07A	A1921	A20-08A	/	w38-03A	1
A1335	A16-27A	/	A32-07A	A1735	A13-23C	/	A16-27A	2
A1339	A14-12C	/	A11-24A	A2032	A31-28A	/	A11-24A	1
A1432	A30-12C	/	A05-28A	A1983	A14-07A	/	A14-07A	1
A1438	A11-24A	/	A10-26A	A1450	w17-07A	/	A11-24A	1
A1450	w17-07A	/	A11-24A	A2042	P03-32A	/	A11-24A	3
A1500	A09-03A	/	A10-NT	A2008	A31-28A	/	A09-03A	1
A1685	A32-07A	/	A19-NT	A1930	A11-12C	/	A32-07A	2
A1703	w44-07A	/	A05-28A	A1432	A30-12C	/	A05-28A	2
A1735	A13-23C	/	A16-27A	A1335	A16-27A	/	A32-07A	1
A1740	A14-07A	/	w17-07A	A1983	A14-07A	/	A14-07A	3
A1743	A14-07A	/	A10-26A	A1980	A14-07A	/	A19-24A	1
A1867	A14-07A	/	A10-26A	A1743	A14-07A	/	A10-26A	3
A1881	A20-08A	/	A32-07A	A1703	w44-07A	/	A05-28A	1
A1921	A20-08A	/	w38-03A	A1102	w38-03A	/	A16-07A	2
A1925	A10-03A	/	A11-24A	A2042	P03-32A	/	A11-24A	3
A1930	A11-12C	/	A32-07A	A1685	A32-07A	/	A19-NT	2
A1983	A14-07A	/	A14-07A	A1743	A14-07A	/	A10-26A	1
O0454	A12-16A	/	A32-07A	O0530	A20-08A	/	A32-07A	2
O0457	A10-18A	/	A30-22I	O0526	A14-11A	/	A30-22I	2
O0458	A16-27A	/	A19-24A	O0531	A20-08A	/	A19-24A	2
O0461	A18-22B	/	A19-03A	O0549	A12-16A	/	A18-22B	1
O0467	A20-08A	/	A32-22J	O0557	A14-27A	/	A32-22J	2
O0475	A16-27A	/	A15-22B	O0533	A14-11A	/	A15-22B	2
O0503	A11-24A	/	A19-24A	O0516	A11-24A	/	A19-10C	2
O0513	A14-27A	/	w17-07A	O0515	A14-27A	/	A19-NT	2
O0515	A14-27A	/	A19-NT	O0513	A14-27A	/	w17-07A	2
O0516	A11-24A	/	A19-10C	O0503	A11-24A	/	A19-24A	2
O0526	A14-11A	/	A30-22I	O0457	A10-18A	/	A30-22I	2
O0530	A20-08A	/	A32-07A	O0454	A12-16A	/	A32-07A	2
O0531	A20-08A	/	A19-24A	O0458	A16-27A	/	A19-24A	2
O0533	A14-11A	/	A15-22B	O0475	A16-27A	/	A15-22B	2
O0549	A12-16A	/	A18-22B	O0461	A18-22B	/	A19-03A	2
O0557	A14-27A	/	A32-22J	O0467	A20-08A	/	A32-22J	2
A2191	A20-08A	/	A05-28A	A1703	A44-07A	/	A05-28A	2
A2202	A13-23E	/	A20-23E	A2301	A19-24A	/	A31-12C	2
A2211	P03-32A	/	w17-07A	A1980	A14-07A	/	A19-24A	2
A2226	A11-24A	/	A11-24A	A1925	A10-03A	/	A11-24A	2
A2227	P03-32A	/	A14-07A	A1867	A14-07A	/	A10-26A	2
A2230	A06-10C	/	A11-24A	A2042	A03-32A	/	A11-24A	2
A2242	w44-07A	/	w17-07A	A2251	A20-08A	/	w17-07A	2
A2245	A20-08A	/	A32-07A	A1335	A16-27A	/	A32-07A	2
A2248	A11-24A	/	A14-07A	A1743	A14-07A	/	A10-26A	2
A2251	A20-08A	/	w17-07A	A1921	A20-08A	/	w38-03A	2
A2256	A06-10C	/	A11-24A	A2226	A11-24A	/	A11-24A	2
A2261	A20-08A	/	A19-24A	A2301	A19-24A	/	A31-12C	2
A2268	A31-11C	/	A16-07A	A1335	A16-27A	/	A32-07A	2
A2283	A14-11A	/	A32-07A	A1980	A14-07A	/	A19-24A	2
A2301	A19-24A	/	A31-12C	A2005	A44-07A	/	A05-28A	2

NT Not typed for class II

eppendorf tube. The tubes were spun (2 min, 15.800 g (14.000 rpm)) and the PBMC were harvested from the interface, and treated as described above. In this thesis PBMC are further referred as lymphocytes.

- lymphocyte absorption

Prior absorption, lymphocytes were washed twice in PBS/0.1% NaN_3. Packed lymphocytes were mixed carefully with serum at a concentration of 10^9 packed cells per ml serum), and were incubated for 1 h at RT, and subsequently 1 h at 4°C. During incubation the mixture was shaken gently. After absorption, the lymphocytes were removed by centrifugation (10 min, 15.800 g (14.000 rpm), 4°C) and the sera were stored at -80°C. The outcome of the 48 immunizations performed to produce typing sera is given in Table 2.

2. Lymphocyte cryopreservation

Freezing procedure (Kleinschuster *et al.* 1979)

Freshly isolated lymphocytes were resuspended in RPMI-1640 with 10% (v/v) FCS (20.10^6 cells/ml) and put on ice for 15 min. Subsequently, ice cold freezing medium (RPMI-1640 10% FCS, 20% DMSO) was added slowly to an equal volume of cell suspension (RPMI-1640 with 10% (v/v) FCS). The suspensions were frozen in aliquot of 1 ml (10.10^6 cells/ml) in polyethylene screw capped tubes (Cryotube, NUNC). After cooling for minimal two hours above the liquid nitrogen, the tubes were transferred to the liquid nitrogen for long time storage.

Table 2. *Result of the 49 immunizations for production of class I and/or class II typing sera. Only the specificity and titer of the immunizations that resulted in a typingserum are given. Difference in class I and class II antigens between donor and recipient is also given.* ☞

Serum ID	Class I incomp. serology A,w,P	activity titer	spec.	Class II incomp. serology Ds	1D-IEF DRBF	activity titer	spec.
A1450	P03	1:4	P03,w44,A10	01,17,14,05	01	1:32,1:64	Ds01,Ds17
O0557	A20	--	--	01,12	06	neat,1:4	Ds01,Ds12
A1102	A20	--	--	12	06	--	--
A1335	A13	--	--	15	11	--	--
A1339	A31	--	--	15	08	--	--
A1432	A14	--	--	02	02	--	--
A1438	w17	1:2	A06,A32	02	02	neat	Ds02
A1500	A31	--	--	03,15	08	--	--
A1685	A11	--	--	01,16,05	05	--	--
A1703	A30	neat	A30	01,16,05	05	--	--
A1735	A32	1:4	A11	02	02	--	--
A1740	--	1:2	A19	--	--	--	--
A1743	A19	1:8	A19	03,10	03	--	--
A1867	--	--	--	--	--	1:8	Ds03
A1881	A05,w44	1:4	P01*	03,15	08	neat	Ds15
A1921	A16	1:16	A16	02	02	neat	Ds02
A1925	P03	1:32	P03	17,14,05	01	--	--
A1930	A19	--	--	--	--	--	--
A1983	A10	1:8	A10	16,04	09	--	--
O0454	A20	1:128	A20	--	06	--	--
O0457	A14	1:64	A13,A14	13	01	--	--
O0458	A20	--	--	01	06	1:32	Ds01
O0461	A12	--	--	08	05	--	--
O0467	A14	>1:32	pos.	03,11	07	1:4	Ds11
O0475	A14	1:2	A05,A18	13	01	--	--
O0503	A19	--	--	01,17,08	06	1:4	Ds08
O0513	A19	1:32	A16	01	06	--	--
O0515	w17	1:16	w17	02	02	--	--
O0516	--	--	--	--	--	--	--
O0526	A10	--	--	01,16,04,07	04	--	--
O0530	A12	1:128	A15,A30	17,08	05	--	--
O0531	A16	1:16	A16	11	07	neat	Ds11
O0533	A16	1:64	A16	03,11	--	1:8	Ds11
O0549	A19	--	--	14,06	05	--	--
A2191	w44	1:64	w44	02	02	1:4	Ds02
A2202	A19,A31	neat	A19	01,16,05,10	03,05	--	--
A2211	A14,A19	--	--	03,10	03	--	--
A2226	A10	--	--	01,17,14,06	05	--	--
A2227	A10	--	--	16,04	09	1:8	Ds04
A2230	P03	1:8	P03,w44	14,05	01	--	--
A2242	A20	--	--	01,12	06	1:16	Ds12
A2245	A16	>1:32	A16+	03,11	07	1:64	Ds11
A2248	A10	--	--	16,04	09	1:4	Ds04
A2251	w38	1:64	w38,(A14)	17,14,06	05	--	--
A2256	--	--	--	--	--	--	--
A2261	A31	1:4	A30	16,05	05	--	--
A2268	A32	1:128	A32	03,11	07	1:16	Ds11
A2283	A19	>1:32	A06	03,10	03	1:4	Ds03
A2301	A05,w44	1:64	P01*	02,15	02,08	--	--

* P01 and P03 are local class I specificities (P01 = w44 + A05 + P03)
pos. Unclear reaction pattern
-- Negative

Recovery procedure

A tube of 1 ml was rapidly thawed in a 37°C water bath, gently rotated until a small crystal of frozen material remained. Immediately the contents was transferred to a 10 ml tube containing 4 ml RPMI-1640 50% FCS at 37°C, gently rotated, and subsequently 4 ml RPMI-1640 10% FCS was added. After centrifugation (10 min, 240 g, 15°C) the cells were suspended in RPMI-1640 10% FCS.

3. Serological typing

* Class I

The antibody-dependent, complement-mediated, double-stain fluorescence lymphocyte microcytotoxicity assay (Bruning et al. 1982)

In short, prior to the class I typing, the lymphocytes were stained with carboxyfluoresceine-di-acetate (Calbiochem): 5.10^6 cells were suspended in 0.5 ml CFDA solution (fresh made, stock 10 mg/ml in acetone p.a. diluted 1:200 in PBS) and incubated for 15 min at 37°C. After staining, the cells were washed twice in PBS and resuspended in RPMI-1640 with 10% (v/v) SERUM PLUS™ (Hazleton); the count was adjusted to $4\text{-}5.10^6$ cell/ml.

Subsequently, 1 μl of serum and 1 μl of the cell suspension were incubated for 30 min at 22°C in the dark. After the addition of 3.3 μl rabbit complement and incubation for 45 min in the dark, 3.3 μl of staining/quenching solution (0.002% propidium iodide and Leitz ink (1/300 suspension w/v) in 5% EDTA, pH7) was added to stain the dead cells. Cytotoxicity was evaluated on an inverted microscope (Zeiss) with epi-illumination immediately after the test. Lysis of the lymphocytes was scored using the following scheme: 1-10% lysis: 1; 11-20% lysis: 2; 21-40% lysis: 4; 41-80% lysis: 6; 81-100% lysis: 8. Scores of 1, 2, and 4 were considered negative, while scores of 6 and 8 were considered positive.

* Class II

The two Color Fluorescence test (Van Rood et al. 1976)

Prior to the class II typing the B-lymphocytes were labelled with rabbit anti-bovine immunoglobulin fluorescein isothiocyanate conjugate (FITC-RABIg, Dakopatts): 10 μl FITC was added to 0.5 ml cell suspension (20.10^6 cell/ml) and the suspension was

incubated for 5 min at 37°C. After labelling, the cells were washed twice in RPMI-1640 and were resuspended in RPMI-1640 with 10% (v/v) SERUM PLUS™ (Hazleton), with the count adjusted to 20.10^6 cell/ml.

Subsequently, 0.5 μl of serum and 0.5 μl of the cell suspension were incubated for 45 min at 22°C in the dark. After the addition of 2.5 μl rabbit complement and incubation for 60 min in the dark, 0.5 μl of haemoglobin/ethidium bromide solution (bovine haemoglobulin solution containing 0.0002% ethidium bromide and 0.5% EDTA, pH7) was added for staining of dead cells. To reduce background lysis caused by aspecific antibodies in the complement, prior to use, the complement was absorbed with a pool of lymphocytes from 4 unrelated cows. Cytotoxicity was evaluated as described for class I typing, except that lysis of B and T cells were scored separately.

4. Biochemical typing (Joosten *et al.* 1988, Joosten *et al.* 1989)

Immuno-precipitation

For metabolic labeling of MHC products freshly isolated lymphocytes were resuspended in S-MEM/20%FCS/1.5% L-Glutamine (10.10^6 cell/ml) and left for 30 min. in an incubator (5% CO_2, 37%C, 100% humidity). For labeling ^{35}S methionine (46 μCi/10.10^6 cell) was added and the cells were left overnight in the incubator (5% CO_2, 37%C, 100% humidity). After labelling the cells were washed once with PBS, and subsequently lysed with a Triton X-114 buffer (0.5% Triton X-114). The suspension was precleared (on ice) with 5 μl normal rabbit serum (NRS), and subsequently with 70 μl of a 10% Staph. aureus suspension (Behring Diagnostics).

For the specific precipitation of MHC class I or class II molecules, 3 μl per 5.10^6 cells mAb (stock solution of 1 mg/ml in PBS) cells was used. For precipitation of class I products mAb W6/32 (SANBIO, seralab) was used. For standard precipitation of the bovine class II products, mAb IL-A21 (DR), TH14B (DR), TH22A5 (DQ) or TH81A (DQ) were used. IL-A21 was a gift from Dr. A.J. Teale, ILRAD, Nairobi, Kenya, the TH-mAb were a gift from Dr. W.C. Davis, Washington State University Pullman, USA). After precipitation the samples were digested overnight at 37°C, with 20 μl neuraminidase type VIII (10 U/ml, Sigma), followed by a second neuraminidase digestion for 3 h at 37°C. After centrifugation (2 min 5.220 g (8000 rpm), RT), the precipitates were kept at -80°C.

One-dimensional isoelectric focusing (1D-IEF)

1D-IEF was performed in vertical polyacrylamide gels. The gels were made using 18 cm x 20 cm glass plates and 1 mm thick PVC spacers. The frame was sealed with 1% agarose and a degassed solution containing 9M urea, 4.5% acrylamide (30% w/v acrylamide and 1.6% w/v bisacrylamide, Millipore), 2% NP-40 (10% stock), ampholines: 4% (v/v) pH 5-7, 1% (v/v) pH 3.5-10, 0.4% (v/v) pH 7-9; LKB, 0.016% ammonium persulphate and 0.08% TEMED was poured between the glass plates. Wells at least 1 cm deep were formed at the upper end of the gel using a 20-tooth comb. After polymerization, the comb and the bottom spacer was removed, the wells were washed with distilled water and the gels were placed in the vertical gel electrophoresis apparatus.

The precipitates were thawed and resuspended in 30 μl IEF-sample buffer (9.5 M urea, 2% NP-40, 2% ampholine pH 3.5-10, 5% ß-mercapto ethanol), left for 30 min at RT, and centrifuged (2 min, 13.800 g (13000 rpm), RT). 20 μl of supernatant was loaded on to the gels, 15 μl of overlay buffer (IEF sample buffer : dd H2O = 1:4) was applied to each sample. The electrode solutions were 0.05 M NaOH for the cathode and 0.02 M H_3PO_4 for the anode. Gels were run for 16.5 h at a constant current of 15 mA, the voltage was limited to 950 V. Gels were fluorographed using DMSO-PPO and were autoradiographed on Kodak X-AR film for 1-3 weeks depending on the labelling efficiency.

For definition of the class I and class II variants, the nomenclature presented at the Fifth International BoLA Workshop was used (Davies *et al.* 1994a, 1994b).
As a negative control an isotype matched mAb recognizing uteroglobulin was used. This mAb lacked the ability to precipitate any radioactive labelled material from cell lysates.

To enable 1D-IEF analysis of DQ-like molecules, the 1D-IEF protocol used for DR typing was modified (chapter 5). The following modifications were applied: 1. an additional preclearing step of 30 minutes using NRS and 70μl of a 10% Staph. aureus suspension was introduced; 2. the composition of the gels was changed to 4.5% acrylamide:bisacrylamide (30:0.8 stock, Millipore), 9M urea, 2% NP-40, 4.5% (w/v) ampholines (4% pH 5-7, 1% pH 3.5-10, 0.4% pH 7-9; LKB); 3. the concentration of ampholine in the sample buffer was changed to 4.8% (v/v) ampholine pH 3.5-10 (+) (Millipore); 4. in addition to ß-mercapto-ethanol, di-thio-threitol (DTT) (to a final concentration of 1.5%) was added to the sample buffer, to enhance the breakage of sulphate bridges; and 5. the gels were run for 18 hours at a constant current of 15 mA and 50 Watt, with the voltage limited to 800 V.

Two-dimensional gel-electrophoresis (2D-IEF) (McMillan et *al.* 1987)

Gel-electrophoresis was as described with the following modifications:

a) ampholines pH 5-7 and pH 3.5-10 were added to a final concentration of 4% and 1%, respectively,

b) an overlay solution was not used, and

c) the IEF gel was run for 14 h at 300 V followed by one hour at 800 V.

SDS-Polyacrylamide gel-electrophoresis (SDS-PAGE)

For the SDS-PAGE, precipitates were run overnight in a 12.5% polyacrylamide gel at 20 mA and a maximum of 120 V. Gels were fluorographed as described above for 1D-IEF. Precipitation was performed with 3 μl ascites.

5. MAILA assay (Mueller-Eckhardt et *al.* 1989)

The assay was performed as described with minor modifications. Flat-bottom microtiter strips (Greiner B.V.) were prepared as described with 100 μl rabbit anti-mouse IgG_{2a} (Serotec code no: SERT 104, 3.1 μg/ml in 50 mM $CaCO_3$) per well. The test procedure was performed with a suspension of fresh lymphocytes ($3x10^6$ cell/ml PBS). HRPO labelled mouse anti bovine IgG_1- (CDI clone code 15.8) and mouse antibovine IgG_2- (CDI clone code 12.5) HRPO mixed 1:1, was used as conjugate. To block aspecific binding of this conjugate to the rabbit anti mouse IgG_{2a} on the plate, the plates were incubated 15 min with wash buffer containing 5% mouse serum prior adding the conjugate. After incubation of two h at 4°C the wells were washed four times and 100 μl of substrate (OPD/UPO mixture, Organon Teknika B.V.), was added. After 10 min the color reaction was stopped by adding 50 μl 4N H_2SO_4. The optical density was read at 492 nm and 620 nm in a titertek photometer.

The test results are expressed by an optical density (OD) ratio, which is calculated by division of the OD value of a sample by the OD of the negative control. The OD ratio $\geq$ 2.0 was considered as a positive reaction. Washbuffer was used as a negative control.

6. Immunofluorescence and flow cytometry (Joling *et al.* 1994).

Freshly isolated lymphocytes were incubated for 30 min, 4°C with 100 μl of mAb diluted in staining medium (RPMI-1640 with 2% FCS and 0.1% NaN_3). After three washes, 100 μl of fluoresceinated goat anti-mouse IgG (GAM-FITC, 1:50, Caltag) was added, and the cells were incubated for an additional 30 min at 4°C. The lymphocytes were washed three times and analyzed by flow cytometry using a fluorescence-activated cell sorter (FACS II, Becton Dickinson). on each experiment 5.000 viable cells were counted and the percentage of the positive cells was calculated. The appropriate controls were included in each experiment to detect any nonspecific labelling.

7. Restriction fragment length polymorphism (RFLP) (Sigurdardóttir *et al.* 1991.)

Genomic DNA was isolated from whole blood (EDTA 15%). The DNA samples were stored at 4°C until use. Samples of 10 μg DNA were digested overnight with the restriction enzymes PvuII at 37°C, or with TaqI at 65°C, both 5 units/μg DNA. The digests were separated by electrophoresis in 0.8% agarose gels and transferred to PallBiodyne A transfer nylon filters. The BRL/Lifetechnologies 1kb ladder was used as a molecular size marker.

The filters were prehybridized in 40% formamide, 0.5M NaCl, 1% SDS, 10% dextran sulphate, 200μg/ml haringsperm DNA at 42°C for 2.5-3h. Radiolabelled probe (^{32}P random priming) was added to the prehybridization mix (activity 1.10^6 cpm/ml). Four bovine class II probes were used:
DQA exon2 (genomic clone W1, XbaI/SstI, 301 bp),
DQA exon3 (genomic clone W1, SstI/KpnI, 420 bp),
DQB exon2 (genomic clone Y1, EcoRI/PstI, 700 bp), and
DQB exon3 (genomic clone Y1, SstI/SstI, 700 bp).

The filters were hybridized overnight at 42°C. After hybridization the filters were washed twice: first in 2 x SSC, 0.5% SDS at RT for 10 min, then 0.5 x SSC, 0.2% SDS, at 56°C-60°C for 10-60 min. The filters were than exposed to Kodac XAR-5 films with Cronex Lightning Plus intensifying screens at -70°C.

The DQA and DQB patterns were ascribed independently, and the nomenclature for DQA and DQB RFLP patterns detected with first and second domain (exon 2 and exon 3) probes was based on the nomenclature presented in the class II report of the Fifth International BoLA Workshop (Davies *et al.* 1994b). The BRL/Lifetechnologies

1kb ladder was used as a molecular size marker.Dehybridization was performed by washing the filter in 0.4M NaOH at 45°C for 5-10 min, and subsequently in 0.1 x SSC, 0.1% SDS, 0.2M Tris pH 7.5 at RT for 2 min.

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Chapter 3

SEROLOGICAL DEFINITION OF BOVINE MHC CLASS II POLYMORPHISM IN HOLSTEIN FRIESIANS

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ABSTRACT

Analysis of the reaction patterns of 57 putative class II MHC alloantisera revealed eighteen B-cell serum clusters, named Ds01 - Ds18. These clusters were grouped together in such a manner that 17 provisional serological class II haplotypes were defined. Segregation in half sib families of these class II haplotypes with serological defined class I and 1D-IEF defined DRB3 types indicated that the 18 clusters identify class II polymorphisms in cattle and can be considered as local specificities.

Keywords: Major Histocompatibility Complex, BoLA, cattle, class II serology.

INTRODUCTION

The bovine major histocompatibility complex (MHC) contains two closely linked regions (Usinger *et al.* 1981), which code for lymphocyte antigens: the BoLA-A and the BoLA-D regions. In the BoLA-D region two loci, DR and DQ are expressed. They encode the class II products, which have shown to be present on B-lymphocytes (Lewin *et al.* 1985) and activated T-lymphocytes (Glass *et al.* 1991).

In cattle two methods are available to identify expressed MHC class II polymorphism: serology and biochemistry. The one-dimensional isoelectric focusing (1D-IEF) is well established for defining polymorphism of the DRB3 gene (Joosten *et al.* 1989, 1990, Davies *et al.* 1994a).

In 1991 three groups reported simultaneously on the production of alloantisera specific for class II bovine lymphocyte antigens. Davies and Antczak (1991) described five local specificities with 10 alloantisera (Dx1, Dx2, Dx3, Dx4, and Dx5), and Arriens *et al.* (1991) defined four local specificities with 13 alloantisera (BeI, BeII, BeIII and BeIV). Williams *et al.* (1991) reported the production of five alloantisera against class II bovine lymphocyte antigens, but no specificities were defined. In the

Fourth International BoLA Workshop, two additional local class II specificities (Dx8 and Dx9) were identified using four sera (Bernoco *et al.* 1991).

Bovine class II alloantisera were raised by allo-immunization. Little is known about the class II haplotypes carried by the donor and the receptor animals (Davies and Antczak 1991, Arriens *et al.* 1991, Williams *et al.* 1991) and consequently, little is known about the complexity of these sera.

In order to gain insight into the complexity of bovine class II sera, locally produced alloantisera were analyzed together with the alloantisera from Arriens, Davies and Williams, which is the largest number of bovine B-cell specific alloantisera ever tested in one laboratory till now. Almost 300 animals were used, among which related as well as unrelated animals. Here we report on the serum cluster analysis and present our local BoLA class II specificities, their distribution in BoLA class II haplotypes and association of these serological defined haplotypes with DRB3 1D-IEF types.

MATERIALS AND METHODS

Animals

For this study, 236 cows housed at the two experimental farms of Wageningen Agricultural University (WAU) were used. These animals were Holstein - Dutch Friesian crosses referred to as Holstein Friesians. They were related as follows: first, 200 of the animals were members of 27 sire families of at least 5 offspring each, thus enabling us to deduce their paternal haplotypes. Second, in 74 instances both the daughter and the mother were typed, enabling us to deduce the maternal-derived haplotypes of the 74 daughters. Furthermore 62 animals were typed as part of the Fifth International BoLA Workshop (WSH) (Davies *et al.* 1994a, 1994b).

Lymphocyte preparation

Lymphocytes were isolated by Ficoll-metrizoate (Lymphoprep, Nycomed) gradient centrifugation of 10 ml heparinized blood (lithium heparin, 14 I.E./ml) samples and were resuspended in RPMI-1640. Prior to typing, the lymphocytes used for class I typing were stained with carboxyfluoresceine-di-acetate (CFDA, Calbiochem): 5.10^6 cells were suspended in 0.5 ml CFDA (fresh made from stock). For the class II typing the B-lymphocytes were labelled with rabbit anti-bovine immunoglobulin fluorescein isothiocyanate conjugate (FITC-RABIg, Dakopatts) (Van Rood *et al.* 1976). After

staining or labelling, the cells were washed twice in RPMI-1640 with 10% (v/v) SERUM PLUS™ (Hazleton) and used immediately in the test.

Reagents

* Class I

A set of 140 class I alloantisera was used. This set consisted of 39 class I typing sera produced locally by planned immunization (skin implants) in Holstein Friesian animals and 101 alloantisera kindly donated by Davies (Ithaca/Beltsville), Lazary (Bern), Østergård (Copenhagen), Spooner (Edinburgh) and Templeton (Texas). All the antisera were characterised during the Fifth International BoLA Workshop (Davies *et al.* 1994b).

* Class II

A set of 49 B-cell specific alloantisera was used (Table 1). This set consisted of: 1) 18 alloantisera produced locally by immunization (skin implants) in class I and class II disparate Holstein Friesians. These sera were absorbed with pooled platelets from selected animals prior to testing in order to remove anti class I antibodies (Van Rood *et al.* 1975). 2) 31 alloantisera kindly donated by Davies (Ithaca/Beltsville), Lazary (Bern) and Spooner (Edinburgh). The 19 sera donated by Davies were raised in Holstein and Angus cattle (Davies and Antczak 1991). The 5 sera donated by Lazary were raised in Simmentaler bulls (Arriens *et al.* 1991). The 7 sera donated by Spooner were raised in pure-bred Friesians (Williams *et al.* 1991). For some of the sera different absorptions or different bleeds were used in parallel, resulting in a test set of 56 B-cell specific alloantisera (Table 1). Furthermore, one monoclonal antibody (mAb) anti human class II (HLA mAb 7.5.10.1 anti HLA DR, DQ, DP), kindly donated by Koning (Leiden) (Koning *et al.* 1984) was used (Table 1). The mAb anti bovine class II IL-A21, kindly donated by Teale (Nairobi) was used as a positive control. Newborn Bovine Serum (NBS; Flow Amstelstad) was used as a negative control. The sera and the mAb were diluted in NBS.

Serological typing

* Class I

The antibody-dependent, complement-mediated, double-stain fluorescence lymphocyte microcytotoxicity assay was performed (Bruning *et al.* 1982). In short, 1μl of serum and 1μl of cell suspension (4000 cells per μl) were incubated for 30 minutes

at 22°C in the dark. After the addition of 3.3μl rabbit complement and incubation for 45 minutes in the dark, 3.3μl of staining/quenching solution (0.002% propidium iodide and Leitz ink (1/300 suspension w/v) in 5% EDTA, pH7) was added to stain the dead cells. Cytotoxicity was evaluated on an inverted microscope (Zeiss) with epi-illumination. Lysis of the lymphocytes was scored using the following scheme: 1-10% lysis: 1; 11-20% lysis: 2; 21-40% lysis: 4; 41-80% lysis: 6; 81-100% lysis: 8. Scores of 1, 2, and 4 were considered negative, while scores of 6 and 8 were considered positive.

* Class II

The two colour fluorescence technique (TCF) was used with minor modifications (Van Rood *et al.* 1976). In short, 0.5μl of serum and 0.5μl of cell suspension (15 - 20,000 cells per μl) were incubated for 45 minutes at 22°C in the dark. After the addition of 2.5μl rabbit complement and incubation for 60 minutes in the dark, 0.5μl of haemoglobin/ethidium bromide solution (bovine haemoglobulin solution containing 0.0002% ethidium bromide and 0.5% EDTA, pH7) was added to stain the dead cells. Prior to use, the complement was absorbed with a pool of lymphocytes from 4 unrelated cows, to reduce background lysis caused by aspecific antibodies in the complement. Cytotoxicity was evaluated as described for class I typing except that lysis of B and T cells were scored separately.

Isoelectric focusing

The IEF protocol of the Fifth International BoLA Workshop (Davies *et al.* 1994a) and the DRB-region Focusing (DRBF) nomenclature proposed at this workshop were used. MHC class II antigens were precipitated with 3μl of the mAb IL-A21 (a gift of Teale, Nairobi).

Statistical analysis

The reactivity patterns of the sera were compared in 2x2 contingency tables. Both the chi square test for independence and the correlation coefficients (*r*-values) were calculated using the Cytofile computer programs developed by Davies (1988).

Table 1. Identification and origin of BoLA alloantisera and HLA mAb.

Serum ID@	Dil.	# Abs	Serum ID Lab of origin	Local spec.	Serum ID@	Dil.	# Abs	Serum ID Lab of origin	Local spec.
B0211-010	neat	-	B0211 07/10/81	Dx1	E0300-011	1:2	l	10552 abs 10041	EDF4
B0580-010	neat	-	B0580 08/12/85	Dx1	E0600-001	1:2	l	10409 abs 10293	EDF4
B0581-012	1:4	-	B0581 11/12/84	Dx1	E1000-001	1:2	l	10409 abs 10186	EDF4
B0581-013	1:8	-	" "	"	E0400-001	1:2	l	10662 abs 102700	EDF6
B0581-023	1:8	-	B0581 11/26/84	Dx1	E0400-002	1:4	l	" "	"
B1156-012	1:4	-	B1156 03/09/89	Dx1	E0500-004	1:16	l	6689 abs 10812	EDF7
B1245-010	neat	-	B1245 02/09/88	Dx1	E1301-000	neat	l	not known	EDF-
B0575-011	1:2	-	B0575 12/17/84	Dx2	E1300-001	1:2	l	" "	"
B0575-012	1:4	-	" "	"	W0557-240	neat	p		Ds01
B0578-011	1:2	-	B0578 12/10/84	Dx2	W1450-355	1:32	p		Ds01
B0695-011	1:2	-	B0695 09/02/85	Dx2	W0458-235	1:32	p		Ds01
B0579-014	1:16	-	B0579 07/29/85	Dx3	W2191-222	1:4	p		Ds02
B0582-014	1:16	-	B0582 02/19/85	Dx3	W1438-141	1:2	p		Ds02
B0781-012	1:4	-	B0781 06/10/85	Dx4	W1921-220	neat	p		Ds02
B1683-026	1:64	-	B1683 11/16/88	Dx4	W1867-323	1:8	p		Ds03
B0303-000	neat	-	B0303 08/17/81	Dx5	W2283-232	1:4	p		Ds03
B0303-010	neat	-	" "	"	W2227-233	1:8	p		Ds04
B0966-000	neat	-	B0966 04/09/87	Dx8	W2248-231	1:2	p		Ds04
B1156-013	1:8	-	B1156 03/09/89	Dx8	W0503-232	1:4	p		Ds08
B1496-013	1:8	-	B1496 12/05/88	Dx8	W2245-236	1:64	p		Ds11
B1751-001	1:2	-	B1751 10/02/89	Dx8	W2268-234	1:16	p		Ds11
B1678-001	1:2	-	B1678 12/27/89	Dx9	W0531-220	neat	p		Ds11
B1679-011	1:2	-	B1679 02/23/90	Dx9	W0467-212	1:4	p		Ds11
B1752-000	neat	-	B1752 02/23/90	Dx9	W0533-223	1:8	p		Ds11
B1752-001	1:2	-	" "	"	W2242-244	1:16	p		Ds12
L0306-013	1:8	-	306	Bel	W0557-242	1:4	p		Ds12
L0339-012	1:4	-	339	Bel	W0557-252	1:4	p		Ds12
L1005-012	1:4	-	1005	BeII	W1881-150	neat	p		Ds15
L1053-012	1:4	-	1053c	BeII	W1881-160	neat	p		Ds15
L1001-012	1:4	-	1001absK2	BeIII	W1450-356	1:64	p		Ds17
E1100-013	1:8	l	6689 abs 10813	EDF2+7	W9997-997	1:100	-	7.5.10.1 anti HLA DRDQDP	Ds18
E0700-003	1:8	l	10095 abs 10041	EDF6					
E0800-002	1:4	l	10409 abs 10015	EDF6	W9997-998	1:200	-	" "	"
E0100-002	1:4	l	10293 abs 10041	EDF4	W9997-999	1:400	-	" "	"

@ Separate ID codes are given to different bleeds, dilutions and absorptions.
B Davies, Ithaca/Beltsville; E Spooner, Edinburgh; L Lazary, Bern; W Nilsson, Wageningen

\# Absorbed with lymphocytes (l); platelets (p); unabsorbed (-).

RESULTS

Anti T-cell reactivity

The anti class II alloantisera were tested against peripheral blood lymphocytes from the 236 WAU animals and the 62 WSH animals in the TCF-test. In only three sera, weak anti T-cell reactivity, which correlated with the class I specificities of these three sera was detected. Sera W0533-233 and W1921-220 showed anti class I A16 reactivity and serum W1881-160 anti class I A05 reactivity. The T cell reactivity indicates incomplete absorption of class I antibodies by platelets. Animals positive for these class I specificities were not excluded from the analysis because the low number of false positive reactions did not influence the clustering of the sera. The other sera did not react with T-cells in the TCF-test.

Serum analysis

The reactivities of the 56 anti B-cell reagents against the panel of the 236 WAU animals were compared in 2x2 contingency tables and the correlation coefficients between the serum pairs were calculated (Table 2). To diminish the effect of genetically identical haplotypes, the cluster analysis was repeated in a group of 104 'unrelated' animals: 74 WAU animals and 30 WSH animals. First and second degree related animals were excluded. Results of the cluster analysis of the group of 'unrelated' animals were practically identical to the analysis of the 236 animals. The individual reaction pattern of the sera and the mAb against the cells were also displayed in serographs (data not shown). On the basis of the *r*-values from the cluster analyses and these serographs, the 56 reagents were grouped into 18 clusters. Each cluster received a local designation (Ds). When a cluster included one or more of the sera from Davies, the number of the cluster name Ds was influenced by the local specificity name from Davies (Dx), i.e. Ds01 is related to Dx1. In addition to the *r*-values between sera, Table 2 shows the *r*-value of each serum with the Ds cluster names that it helps define.

Eleven clusters (Ds01, Ds02, Ds03, Ds04, Ds06, Ds08, Ds09, Ds10, Ds11, Ds12, and Ds17) were defined using sera with strong positive correlations produced by at least two different animals. The assignment of Ds06 was based on three sera: E0100-002, E0300-011 and E1000-001. Serum E1000-001 originated from animal 10409. To confirm Ds06 assignments a different absorption of the serum from animal 10409 (E0600-001) was used.

Table 2. Correlation coefficients (R>.30) between sera and their proposed cluster names based on 104 "unrelated" animals.

1. Ds01							
2. W0557-240	.96						
3. W1450-355	.96	.92					
4. B0580-010	.90	.85	.89				
5. B1156-012	.88	.88	.84	.78			
6. W0458-235	.88	.84	.85	.79	.80		
7. L0306-013	.81	.81	.85	.73	.70	.71	
8. L0339-012	.68	.72	.68	.61	.69	.62	.78
	1	2	3	4	5	6	7

1. Ds02						
2. W1438-130	1.					
3. B0575-012	.97	.97				
4. B0695-011	.95	.95	.92			
5. B0578-011	.93	.93	.90	.87		
6. W2191-222	.92	.92	.89	.92	.85	
7. W1921-220	.77	.77	.75	.77	.70	.76
	1	2	3	4	5	6

1. Ds03							
2. E1100-013	.96						
3. L1005-012	.94	.94					
4. L1053-012	.92	.88	.86				
5. B0582-014	.89	.84	.83	.82			
6. W1867-323	.82	.81	.80	.75	.92		
7. B0579-014	.81	.79	.75	.75	.91	.91	
8. W2283-232	.80	.78	.81	.78	.71	.69	.65
	1	2	3	4	5	6	7

1. Ds04						
2. W2227-233	1.					
3. B1683-026	1.	1.				
4. B0781-012	1.	1.	1.			
5. E0700-003	.97	.97	.97	.97		
6. W2248-231	.97	.97	.97	.97	.94	
7. E0800-002	.97	.97	.97	.97	.93	.93
	1	2	3	4	5	6

1. Ds05			
2. B0303-010	1.		
3. B0303-000	.81	.81	
	1	2	

1. Ds06				
2. E1000-001	1.			
3. E0100-002	.95	.94		
4. E0300-011	.94	.94	.89	
5. E0600-001	.81	.81	.86	.76
	1	2	3	4

1. Ds07		
2. E0400-002	.96	
3. E0400-001	.55	.52
	1	2

1. Ds08					
2. B1496-013	.87				
3. B1751-001	.83	.88			
4. W0503-232	.77	.66	.61		
5. B1156-013	.75	.64	.71	.68	
6. B0966-004	.63	.56	.46	.61	.52
	1	2	3	4	5

1. Ds09				
2. B1752-001	.88			
3. B1679-011	.83	.77		
4. B1678-001	.64	.53	.59	
5. B1752-000	.51	.42	.53	.71
	1	2	3	4

1. Ds10		
2. E0500-004	1	
3. W2283-232	.83	.83
	1	2

1. Ds11					
2. W2245-236	1.				
3. W0467-212	.85	.85			
4. W0531-220	.82	.82	.82		
5. W0533-223	.78	.78	.66	.63	
6. W2268-234	.74	.74	.62	.60	.56
	1	2	3	4	5

1. Ds12				
2. W2242-244	.88			
3. W0557-252	.83	.78		
4. W0557-242	.83	.73	.82	
	1	2	3	

1. Ds13		
2. L1001-012	.97	
3. B0575-011	.39	.42
	1	2

1. Ds14			
2. B0581-023	.97		
3. B0581-012	.88	.85	
4. B0581-013	.86	.82	.82
	1	2	3

1. Ds15		
2. W1881-150	1.	
3. W1881-160	.79	.79
	1	2

1. Ds16		
2. E1301-001	.88	
3. E1300-000	.66	.58
	1	2

1. Ds17			
2. W1450-356	.87		
3. B0211-010	.81	.67	
4. B1245-010	.75	.65	.63
	1	2	3

1. Ds18			
2. W9997-998	.92		
3. W9997-997	.92	.84	
4. W9997-999	.61	.36	.36
	1	2	3

The cluster Ds09 was based on three sera. Two sera (B1679-011 and B1752-001) were well correlated and were used to define the Ds09 cluster. The third serum (B1678-001) was a broad serum, correlating better with B1752-000 (B1752 used undiluted) than B1752-001. Sera B1678-001 and B1752-000 were only used to confirm Ds09 assignments. The cluster Ds10 was based on two sera, E0500-004 and W2283-232, of which the latter also had Ds03 reactivity.

The assignment of Ds12 was based on two sera: W0557-242 and W2242-244. A different absorption of the serum W0557 (W0557-252) was used to confirm Ds12 assignments. The cluster Ds13 was based on two sera, L1001-012 and a tail of the Ds02 serum B575-011, resulting in a low *r*-value between these sera. The remaining clusters (Ds05, Ds07, Ds14, Ds15, Ds16) were each based on different dilutions or absorptions or bleeds of a serum from a single animal. Therefore, *r*-values for these sera, shown in Table 2, can be considered to be an internal quality control. The sera defining the clusters Ds07 and Ds16 were only used on part of the animals.

The anti HLA DR,DP,DQ mAb 7.5.10.1 was tested in parallel with the alloantisera. This antibody was found to react with only a few animals. The reaction pattern was designated Ds18. Duplicate typing using different antibody dilutions was only performed on part of the animals.

In this study we have compared sera from different laboratories each defining local specificities. Sera recognising Bel and Dx1 were strongly positively correlated with each other and with the other three sera used to define Ds01. Anti Bell and Dx3 sera correlated strongly with each other and the other three sera used to define Ds03. The BelII specificity, for which there was only one serum, was confirmed by reactivity in one of the Dx2 sera from Davies and both sera were used to define Ds13.

Cluster associations

To reveal associations of the 18 assigned clusters in the 236 WAU animals, cluster analysis of the 18 assigned clusters was performed. The *r*-values calculated with the 'unrelated' WAU animals are given in Table 3. Furthermore, cluster associations were displayed in serographs showing the distribution of the clusters among the animals. Because the correlation coefficients calculated with 74 'unrelated' WAU animals were practically identical to those calculated with all 236 WAU animals, in Figures 1 - 5 only the distribution of the clusters in 236 WAU animals is shown. All of the clusters are shown in Figure 1.

Both Ds01 and Ds03 are broadly reactive. Seven clusters are associated with Ds01: Ds05, Ds06, Ds08, Ds09, Ds12, Ds14 and Ds17 (Figure 2 and Table 3). The

Table 3. Correlation coefficients (R >.30) between the 18 clusters based on 74 'unrelated' WAU animals.

CLUSTER ID	09	05	12	08	06	14	17	01	07	04	16	11	15	18	10	03	02	13
Ds09																		
Ds05	.																	
Ds12	.	.																
Ds08	.	.	.															
Ds06	.	.	.	.														
Ds14	.	.	.	.	.64													
Ds17	.	.	.	.41	.43	.55												
Ds01	.	.	.45	.	.	.31	.50											
Ds07	.	.	.	.	.	.	.	.										
Ds04	.	.	.	.	.	.	.	.	.50									
Ds16	.	.	.	.	.	.	.	.	.34	.55								
Ds11	.	.	.	.	.	.	.	.	.	.	.							
Ds15	.	.	.	.	.	.	.	.	.	.	.	.						
Ds18	.	.	.	.	.	.	.	.	.	.	.	.	.					
Ds10	.	.	.	.	.	.	.	.	.	.	.	.	.	.45				
Ds03	.	.	.	.	.	.	.	.	.	.	.	.	.	.37	.69			
Ds02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Ds13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

CLUSTER ID

cluster Ds06 is almost completely included in Ds14. Furthermore, Ds14 and Ds08 are negatively associated, but are both included in Ds17. Ds17 is almost completely included in Ds01. Figure 3 shows the distribution of Ds05, Ds06, Ds08, Ds09 and Ds12 next to Ds01. Four clusters are associated with Ds03: Ds10, Ds11, Ds15 and Ds18 (Figure 4 and Table 3). The cluster Ds18 is almost completely included in Ds10. The cluster Ds10, Ds11 and Ds15 are negatively associated but are all included in Ds03.

The five remaining clusters Ds02, Ds04, Ds13, Ds07 and Ds16 were neither included in nor closely related to Ds01 or Ds03. Clusters Ds02, Ds04 and Ds13 show unique reactivity patterns and no subtypes of Ds02 or Ds13 were defined. Positive correlations were seen between Ds16, Ds07 and Ds04 (Figure 5 and Table 3), and between Ds16 and Ds05 (Figure 5). The Ds04 cluster is associated with Ds16, and Ds07 was found in over 75% of the Ds04 positive Ds01 positive animals (15 of 19,

$r = .63$). No animals negative for the five clusters, Ds01, Ds03, Ds02, Ds04, and Ds13, were observed; animals negative for Ds01, Ds02 and Ds03 were positive for Ds04 and/or for Ds13.

Haplotype definition

As can be seen in Figure 1 per animal the number of clusters of which sera reacted with cells varied widely. Clusters that often occurred together in the WAU animals were grouped as provisional haplotypes. In this way 17 serological class II haplotypes could be defined. Serologically defined class II haplotypes are designated by their broadest cluster name followed by the other cluster name in order of decreasing frequency. For example, if an animal was positive for Ds06, then it always was positive for Ds14, Ds17 and Ds01 and it was assigned the Ds01-17-14-06 haplotype. All the serological class II haplotypes were defined by more than one cluster, except for the haplotypes defined by the Ds02 and Ds13. The class II haplotypes were verified by segregation in the paternal and maternal families. All animals used for screening of the anti B-cell sera were typed for class I by serology as well as for class II DRB3 by 1D-IEF. The DRB3 1D-IEF typing supported the definition of class II haplotypes, and because the bovine class I and class II genes are tightly linked (Usinger *et al.* 1981), the class I typing of the animals was used as a marker for the segregation of the class II haplotypes. Most class II haplotypes occurred with only a limited number of class I types. In the 236 WAU animals 19 different class I alleles and 19 class II haplotypes, comprising 32 class I + II haplotypes, were identified (data not shown).

The 19 class II haplotypes found in the 236 WAU animals, and the number of times each haplotype was inherited from the father or the mother is given set out in Table 4. Reliable haplotype assignment of Ds15 was not always possible in Ds03 DRBF11 positive homozygous animals. Ds18 appeared to be restricted to Ds03-10 DRBF03 positive animals. However duplicate testing of the mAb defining Ds18 was

Figure 1. Graphic representation of the distribution of all the clusters (N = 236).

N Number of animals tested
■ Indicates positive reaction for a major cluster
\+ Indicates positive reaction
□ Indicates negative reaction
\- Not tested

☞

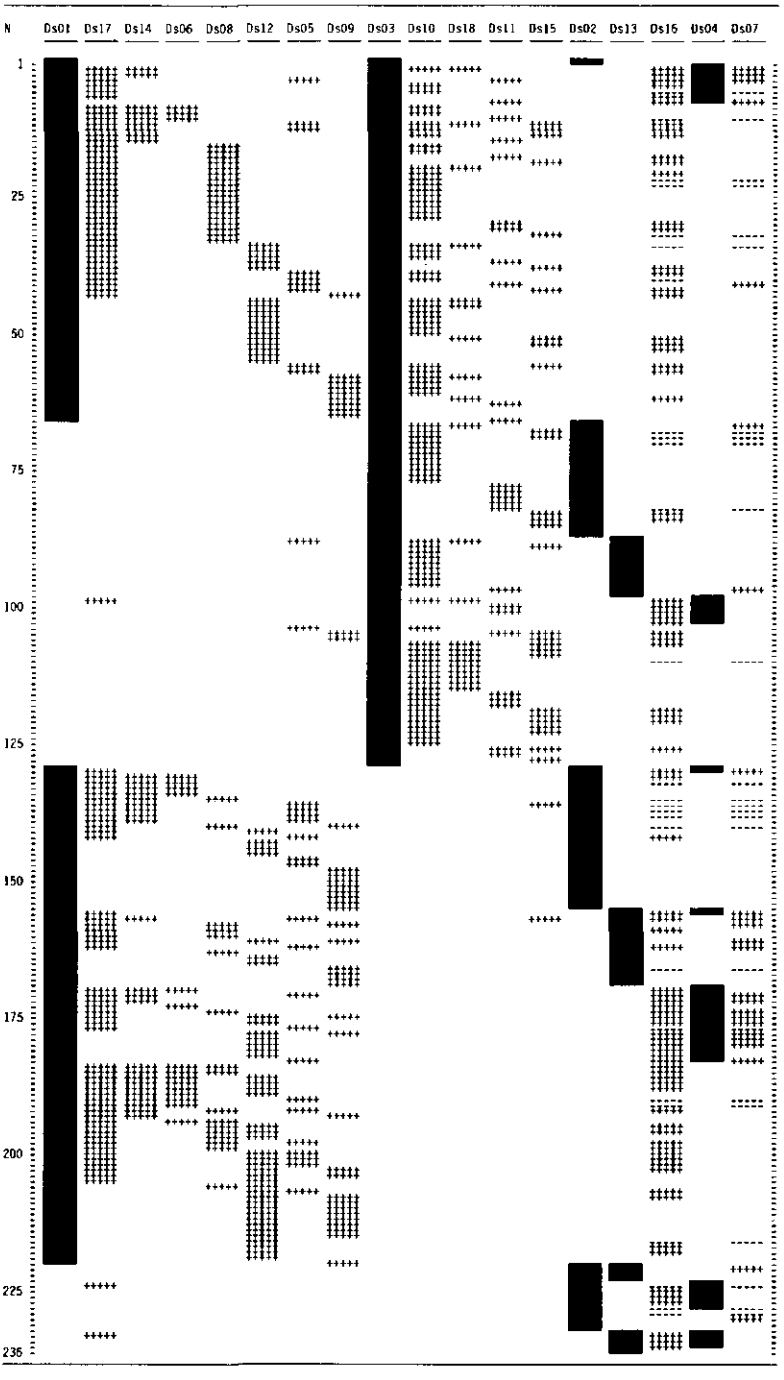
N
Ds01
Ds17
Ds14
Ds06
Ds08
Ds12
Ds05
Ds09
Ds03
Ds10
Ds18
Ds11
Ds15
Ds02
Ds13
Ds16
Ds04
Ds07
1
25
50
75
100
125
150
175
200
225
235

Figure 2. Graphic representation of the distribution of the clusters Ds01, Ds17, Ds14, Ds06, Ds08, Ds12, Ds05 and Ds09 (N = 236).
N Number of animals tested
■ Indicates positive reaction
□ Indicates negative reaction

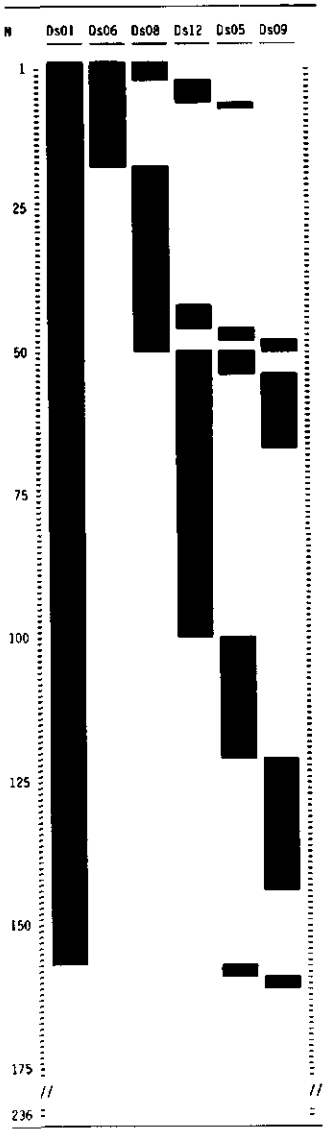

Figure 3. Graphic representation of the distribution of the clusters Ds01, Ds06, Ds08, Ds12, Ds05 and Ds09 (N = 236). For legend see Figure 2.

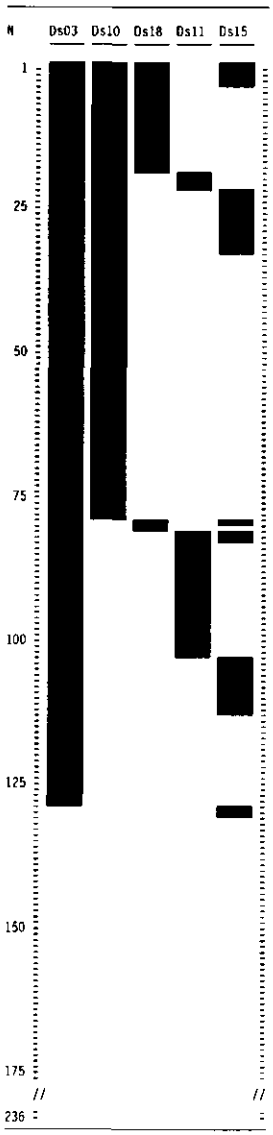

Figure 4. Graphic representation of the distribution of the clusters Ds03, Ds10, Ds18, Ds11 and Ds15 (N = 236). For legend see Figure 2.

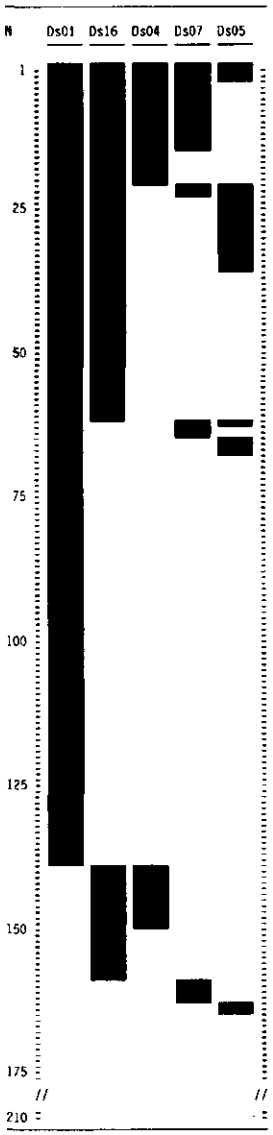

Figure 5. Graphic representation of the distribution of the clusters Ds01, Ds16, Ds04, Ds07 and Ds05 (N = 210). For legend see Figure 2.

only performed on part of the panel, so Ds18 assignment could not be confirmed by segregation. False negative assignments were seen with Ds07, Ds10, Ds15 and Ds16 (data not shown), whereas not assignable reactivity was seen with Ds07, Ds10 and Ds15, and above all with Ds16 and Ds17. Ds16 is often found to segregate with Ds04 or Ds05. However, in 39 animals that were Ds16 positive and Ds04/Ds05 negative, Ds16 did not clearly segregate. Likewise Ds17 did not clearly segregate in Ds17 positive and Ds14/Ds08 negative animals.

Table 4. Class II haplotype segregation in half-sib families.

Class II haplotype serology	1D-IEF	paternal (N = 200) N_{pos}	maternal (N = 73) N_{pos}
Ds01-09	DRBF07	11	4
Ds09	DRBF07	-	1
Ds01-12	DRBF06	44	1
Ds01-05	DRBF08	-	1
Ds01-16-05	DRBF05	8	4
Ds01-17-14-05	DRBF01	6	1
Ds01-17-08	DRBF05	10	4
Ds01-17-08	DRBF06	4	-
Ds01-17-14	DRBF06	4	-
Ds01-17-14-06	DRBF05	8	6
Ds01-16-04-07	DRBF04	-	1
Ds16-04	DRBF09	4	2
Ds02	DRBF02	14	17
Ds03	DRBF11	4	1
Ds03-10	DRBF03	47	10
Ds03-11	DRBF07	8	7
Ds03-15	DRBF08	-	9
Ds03-15	DRBF11	7	1
Ds13	DRBF01	21	3

In Table 5 the segregation of the inferred class I + II haplotypes of the 27 sires is shown. For two sires (numbers 41 and 46) only one haplotype could be inferred because all their offspring received the same paternal haplotype. It is not known if these sires are BoLA homozygote.

Table 5. Class I + class II genotypes of 27 sires as deduced by segregation of 200 informative offspring.

Sire ID	first haplotype (H1) Class I	Class II		/ second haplotype (H2) Class I	Class II		number transmitted H1	H2
23	w44	Ds02	DRBF02	/ A20	Ds01-12	DRBF06	4	3
26	P03@	Ds01-17-14-05	DRBF01	/ A14	Ds02	DRBF02	4	5
33	A11	Ds01-16-05	DRBF05	/ A20	Ds01-12	DRBF06	4	2
35	A10	Ds16-04	DRBF09	/ A15	Ds01-09	DRBF07	4	1
37	A11	Ds03-10	DRBF03	/ A19	Ds03-10	DRBF03	6	1
41	A20	Ds01-12	DRBF06	/ ND	ND	ND	6	0
42	A19	Ds03-10	DRBF03	/ A11	Ds03-10	DRBF03	3	4
43	A13	Ds03-15	DRBF11	/ A14	Ds03-11	DRBF07	5	3
44	A11	Ds03-10	DRBF03	/ A19	Ds13	DRBF01	5	3
45	A19	Ds03-10	DRBF03	/ A10	Ds01-17-14-06	DRBF05	3	4
46	A20	Ds01-12	DRBF06	/ ND	ND	ND	8	0
47	A12	Ds01-17-08	DRBF05	/ A13	Ds03	DRBF11	1	4
48	A11	Ds01-16-05	DRBF05	/ A11	Ds03-10	DRBF03	4	5
49	A14	Ds13	DRBF01	/ A20	Ds01-12	DRBF06	4	2
50	A12	Ds01-17-08	DRBF05	/ A10	Ds01-17-14-06	DRBF05	2	4
51	A19	Ds03-10	DRBF03	/ A31	Ds01-17-14	DRBF06	7	4
52	A12	Ds01-17-08	DRBF05	/ A06	Ds01-17-08	DRBF06	4	4
53	A20	Ds01-12	DRBF06	/ A14	Ds13	DRBF01	4	4
54	w44	Ds02	DRBF02	/ A20	Ds01-12	DRBF06	4	3(+1*)
55	A14	Ds02	DRBF02	/ A19	Ds03-10	DRBF03	1	5
56	A20	Ds01-12	DRBF06	/ A14	Ds13	DRBF01	2	6
57	P03@	Ds01-17-14-05	DRBF01	/ A15	Ds01-09	DRBF07	2	7
58	A20	Ds01-12	DRBF06	/ A12	Ds01-17-08	DRBF05	4	3
59	A20	Ds01-12	DRBF06	/ A12	Ds03-10	DRBF03	4	4
60	A20	Ds01-12	DRBF06	/ A15	Ds01-09	DRBF07	5	3
61	A13	Ds03-15	DRBF11	/ A16	Ds03-11	DRBF07	2	5
62	A19	Ds03-10	DRBF03	/ A14	Ds13	DRBF01	4	4

ND Not detected
* one recombinant haplotype: w44 Ds01-12
@ P03 is a local class I specificity

Among the 27 sires, 13 class I alleles and 15 class II haplotypes were detected, these were combined to form 20 class I + II haplotypes. One cross-over between class I and class II was detected among the paternal haplotypes inherited by the 200 offspring.

Association of different 1D-IEF types with the same serological class II haplotype occur, e.g. Ds01-17-08 is associated with DRBF05 and with DRBF06 (sire 53,

Table 5). Next to that in several cases different serological class II haplotypes appeared to be associated with the same 1D-IEF type (Table 4, Table 5). Two examples of correlation between these serological class II haplotypes and 1D-IEF DRB3 types as revealed with 65 'unrelated' WAU animals are shown in Table 6.

Table 6. Two examples of correlation between serological defined class II haplotypes and 1D-IEF types, based on 65 'unrelated' WAU animals.

Serology	1D-IEF	++	+-	-+	--	N	r
Ds01-17-14-05	DRBF01	3	0	8	54	65	0.49
Ds13	DRBF01	9	0	2	54	65	0.89
Ds01-17-14-05 / Ds13	DRBF01	11	0	0	54	65	1.00
Ds01-09	DRBF07	9	0	4	52	65	0.80
Ds03-11	DRBF07	5	0	8	52	65	0.58
Ds01-09 / Ds03-11	DRBF07	13	0	0	52	65	1.00

DISCUSSION

The reactivity of the anti B-cell reagents was tested in the TCF-test. Previous studies involving BoLA class II serology were performed using the microcytotoxicity assay with a B-cell enriched suspension (Davies and Antczak 1991, Arriens *et al.* 1991, Williams et *al.* 1991). We preferred the TCF-test as screening method, because of two major advantages: 1) simultaneous detection of the anti B-cell and T-cell reactivity of the serum; and 2) increased sensitivity of the test system due to, a) prolonged incubation, and b) staining of the lysed cells with ethidium bromide instead of staining with eosin (Nieman *et al.* 1983). The TCF-test however, is more laborious than the microcytotoxicity assay combined with a B-cell enriched suspension, and therefore, once the screening of class II specific polymorphic sera is made, the latter might have preference for typing of a large group of animals. In the class I testing the double-stain fluorescence instead of staining with eosin was used, because this method of dying not only increases the sensitivity of the test system, but also facilitates the reading of the test.

The 57 anti B-cell reagents selected for this study reacted specifically with B-lymphocytes. These sera are thought to react with bovine class II molecules for the following reasons. The serum clusters defining Ds01, Ds02, Ds03, Ds04, Ds05, Ds08, Ds09, Ds13, Ds14 and Ds17 contained sera from Davies and/or Arriens who demonstrated that some of their sera immunoprecipitated class II molecules (Arriens *et al.* 1991, Williams *et al.* 1991). The clusters defining Ds06, Ds07, Ds10 and Ds16 contained sera from Williams who showed strong association between his sera and polymorphism of DRB3 detected by 1D-IEF (Williams *et al.* 1991). The three remaining clusters defined with alloantisera are based on locally produced sera and associate with the serum clusters Ds01 (Ds12) and Ds03 (Ds11 and Ds15). The Ds18 specificity is defined by a mouse anti-human mAb directed against a monomorphic HLA class II epitope. Ds18 has a restricted distribution in cattle and appears to be associated with the Ds03-10 DRBF03 haplotype. This is the first report of a monomorphic anti-human class II mAb showing restricted reactivity with bovine B-lymphocytes. An immunoprecipitation study with this mAb is needed to confirm that it reacts with an epitope on a bovine class II molecule.

In Figure 2, Figure 3 and Figure 4 incomplete inclusions were seen, e.g. of Ds17 into Ds01, Ds06 into Ds14, Ds15 into Ds03 and Ds18 into Ds10. The non-included fraction may represent additional low frequency specificity. Next to that, Ds16 and Ds17 showed false positive assignment, probably because the sera defining these clusters contain antibodies against both BoLA and non-BoLA determinants. However in this stage of bovine class II serology, with the limited number of class II sera available, we have used all the clusters, in spite of the poor definition of some of them, for haplotype definition. They all provide essential information in detecting class II variation by serology, e.g. Ds16 and Ds17 are useful in distinction between Ds01-05 DRBF05 (Ds16-positive), Ds01-05 DRBF01 (Ds17-positive) and Ds01-05 DRBF08 (Ds16- and Ds17-negative) respectively.

Reliability of the serological class II assignments was assessed by segregation. The serological class II haplotype definition was supported by 1D-IEF DRB typing. Furthermore the serological class I typing was used as a marker for the segregation of the total class II haplotypes. Close linkage between the BoLA-A locus and the BoLA-D region (Usinger *et al.* 1981), was confirmed by the observation that each class I allele was associated with a limited number of class II haplotypes, only 20 class I + II haplotypes occurred in the 27 sires (Table 5). As the clusters segregated so well with the serological class I and 1D-IEF class II DRB3 types, we considered the clusters as local specificities.

1D-IEF defined DRB polymorphism can function as a marker for the class II haplotype definition, but only if DR and DQ are as tightly linked as stated by Sigurdardóttir *et al.* (1988). The class II serotyping reagents available at present enable a more extensive discrimination of haplotypes than ID-IEF defining DRB polymorphism, 17 serological haplotypes versus 10 1D-IEF DRB types.

Obviously, it should be taken into account that MHC class II products with different serological specificities can focus at identical position in the gel. On the other hand, it can also be that a single DRBF type is associated with different DQ alleles. As yet, we do not know if the 18 putative serological specificities described here define individual BoLA DR or DQ allelic products, or combinations of them. The alloantisera defining these class II specificities are mixtures of antibodies resulting from an immune response directed against a complex class II haplotype, comprised of products of one DR gene and one or two DQ genes. Studies in humans have shown that class II HLA alloantisera often contain mixtures of anti HLA-DR and DQ or mixtures of antibodies against products of several DR loci. Moreover, different DR and DQ allelic products share epitopes that can be recognised by antibodies. Therefore our local bovine class II specificities must, for the time being, be considered as haplotype sero-typing until further studies such as absorption have been carried out.

Since serotyping is cheaper and less laborious than 1D-IEF, once having compiled a panel of class II typing sera, serotyping is favourable for detecting class II haplotypes on product level in population studies. This paper shows that, even with the limited amount of bovine class II sera available at this moment, serology can be used for definition of expressed class II polymorphism (DR and DQ) in Holstein Friesians, especially if combined with class I serology. However, to reveal class II polymorphism of one locus, at this stage class II serology is not sufficient and has to be combined with selective 1D-IEF typing.

When the sera described here were used to type the 62 animals from the Fifth International BoLA Workshop (Davies *et al.* 1994a), five of the 18 local specificities presented in this paper were given official workshop designations: Ds01 = Dw1, Ds02 = Dw2, Ds03 = Dw3, Ds04 = Dw4 and Ds08 = Dw8. Next to that, seven specificities were given workshop cluster names: Ds05 = Dc5, Ds06 = Dc6, Ds07 = Dc7, Ds09 = Dc9 and Ds11 = Dc11, Ds12 = Dc12 and Ds13 = Dc13. Furthermore, 12 of the 19 class II haplotypes described in the WAU animals were detected, whereas also more novel combinations of the serum clusters were found

indicating that the associations between class II specificities as well as their DR and DQ encoding genes may be breed or even herd specific.

In conclusion, serological definition of bovine class II polymorphism is possible. The identification of seventeen serological class II haplotypes, all confirmed by segregation analysis, demonstrates the extensive polymorphism of expressed bovine class II genes. Further studies comparing BoLA class II polymorphism detected by serology with class II polymorphism detected by other typing methods are in progress.

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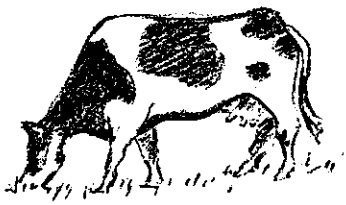

Chapter 4

COMPLEXITY OF THE BOVINE MHC CLASS II SPECIFICITY DW3 AS DEFINED BY ALLOANTISERA

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Ph.R. Nilsson, J.W. van 't Klooster, J.J. van der Poel, W.C. Davis and G.M.Th. Schreuder

ABSTRACT

Alloantisera related to the bovine major histocompatibility complex (MHC) class II specificity Dw3 were investigated by cross-absorption experiments and by application of the monoclonal antibody-specific immobilization of lymphocyte antigen assay (MAILA). The absorption study revealed antibodies specific for an antigenic determinant shared by all Ds03(=Dw3) positive animals, and several other antibody populations recognizing the locally defined specificities Ds10, Ds11 and Ds15, that are closely associated with Ds03. The results of the MAILA-assay indicate that the Ds03 specificity is probably encoded by DQ, whereas specificities Ds10 and Ds11 are more closely associated with DR molecules. The data presented here provide the first evidence that bovine DR and DQ specificities can be identified separately by serological methods using alloimmune antisera.

Keywords: BoLA, class II serology, complexity Dw3

INTRODUCTION

In cattle, major histocompatibility complex (MHC) class II specific alloantisera have been raised by planned immunization through injection of leucocytes or by subcutaneous skin implantation. Two strategies have been used. Firstly: immunizations between 'class I identical class II non-identical' animals (Arriens *et al.* 1991, Williams *et al.* 1991, Davies and Antczak 1991). Secondly: immunizations between 'class I and class II non-identical' animals, with subsequent platelet absorption of the sera to remove the anti class I reactivity (Cwik *et al.* 1979, Newman *et al.* 1982, Mackie and Stear 1990, 1992). Irrespective of the strategy used, only a limited number of class II typing sera was produced.

Despite the difficulties in production, five class II specificities were defined serologically in the Fifth International Bovine Lymphocyte Antigen (BoLA) Workshop.

One of the workshop specificities, BoLA-Dw3, appeared to be associated with seven different isoelectric focusing (1D-IEF) defined DRB3 types (Davies *et al.* 1994a). BoLA-Dw3 may either represent an allospecificity that is shared among several allelic products of the DRB3 gene, or be carried by allelic products of other class II genes associated with the DRB3 haplotype e.g. DQ.

In cattle little is known about the expression of the class II molecules on the different haplotypes. Also information is lacking concerning the immunogenic difference between donor and recipients used for production of class II alloantisera. Consequently little is known about the complexity of these sera, and it is likely that they contain different antibodies against several allelic products.

To study the heterogeneity of Dw3 alloantisera, several animals were immunized with emphasis on three of the 1D-IEF defined (DRBF) Dw3 associated subtypes: Dw3 DRBF03, Dw3 DRBF07 and Dw3 DRBF08 and cross-absorption experiments were applied for characterization of the produced antisera. Subsequently the monoclonal antibody-specific immobilization of lymphocyte antigen (MAILA) assay (Mueller-Eckhardt *et al.* 1989) was applied for further analysis, as this assay enables discrimination between antibodies against the DR and DQ molecules separately.

Unravelling the heterogeneity of Dw3 alloantisera may give insight, not only in the composition of these class II sera, but also in the composition of the immunogenic determinants of BoLA-Dw3 associated haplotypes.

MATERIALS AND METHODS

Animals

The animals used in this study were part of a group of Holstein Friesian cows from the Wageningen Agricultural University (WAU). All animals were typed serologically for class I and class II, and biochemically by isoelectric focusing (1D-IEF) for DRB3. The age of the animals at the moment of first immunization ranged from 9 months to 3 year and 8 months. Three pairs of animals used for immunization (W0467 - W0557, W0531 - W0458, W0533 - W0475) were mother - daughter combinations.

Typing techniques

- Serology. The antibody-dependent complement mediated double-stain fluorescence lymphocyte microcytotoxicity assay was used for class I typing (Bruning *et al.* 1982).

The Fifth International BoLA Workshop (Davies *et al.* 1994b) nomenclature is used for class I (A,w). Class II typing was performed with the two colour fluorescence technique (TCF) (Van Rood *et al.* 1976) and local designation was used for identification of specificities (Ds) (Nilsson *et al.* 1994). One of the sera used to study Ds03 (B0579) was originally defined Dx3 specific (Davies and Antczak 1991), however in the Fifth International BoLA Workshop (Davies *et al.* 1994a), both Dx3 and Ds03 were assigned official workshop designation Dw3, thus Ds03 = Dw3 = Dx3. Initially class II typing was performed with a limited set of sera. Recently all animals have been retyped with a more extensive set of 56 class II alloantisera.

- Isoelectric focusing. The method used was described by Joosten *et al.* (1989) using the monoclonal antibody (mAb) IL-A21 (a gift of Dr. A.J. Teale, ILRAD, Nairobi) to precipitate BoLA DR molecules. The D-region focusing (DRBF) nomenclature proposed by the Fifth International BoLA Workshop (Davies *et al.* 1994a) was applied.

Immunization

Immunization for Ds03 related alloantisera was performed by skin implantation (Pringnitz *et al.* 1982) between animals differing in class I and class II specificities. Selection of donor recipient pairs: based on the initial serological and biochemical typing animals were chosen to differ for Ds03 and/or DRBF03, DRBF07 and DRBF08 respectively. The seven selected pairs are shown in Table 1. Skin implantation was repeated after one month, except for one animal (W1881). Serum was collected prior to immunization and 18-21 days after the second immunization. Since the sera B0579 and E6689 have been used in the lymphocyte absorption study the available information as given by Davies (Davies and Antczak 1991, Davies *et al.* 1992) and Williams (AFRC, Edinburgh, personal communication) is included in Table 1.

Serum absorption

To remove class I specific antibodies serum absorption was performed, using platelets or lymphocytes.

- platelet isolation

The platelets were separated by differential centrifugation of freshly collected blood, using 15% EDTA (10:1 v/v) as anti-coagulate. Blood was diluted with an equal volume of PBS/1%EDTA pH7.4, and centrifuged (10 min, 600 g, RT, no brake). The

Table 1. Class I and class II genotypes of recipient and donor animals involved in the production of Ds03 related alloantisera.

RECIPIENT							DONOR						
ID	Cl I A	Cl II Ds	IEF DRBF	/ Cl I / A	Cl II Ds	IEF DRBF	ID	Cl I A	Cl II Ds	IEF DRBF	/ Cl I / A	Cl II Ds	IEF DRBF
W1881	20	01-12	06	/ 32	02	02	W1703	05	03-15	08	/ 44	02	02
W0467	20	01-12	06	/ 32	##	05	W0557	14	03-11	07	/ 32	##	05
W0531	20	01	11	/ 19	03-10	03	W0458	16	03-11	07	/ 19	03-10	03
W0533	14	13	01	/ 15	01-09	07	W0475	16	03-11	07	/ 15	01-09	07
W2245	20	01-12	06	/ 32	02	02	W1335	16	03-11	07	/ 32	02	02
W2268	31	01-14	06	/ 16	02	02	W1335	16	03-11	07	/ 32	02	02
W2283	32	13	01	/ 14	02	02	W1980	19	03-10	03	/ 14	02	02
E6689*	18	NT	@@	/ 15	NT	07	E10185	18	NT	03	/ 15	NT	07
B0579*	06	02	02	/ 06	01	06	B0505	06	03	03	/ 06	01	06

* Immunization performed by Williams *et al.* 1991 (E) and Davies & Antczak 1991 (B)
NT Not typed by class II serology
Sero-type not detected with the available class II sera (= blank)
@@ Local defined type: EDF12

supernatant (platelet rich plasma) was collected carefully and the procedure was repeated one more time. The platelets were pelleted by centrifugation of the platelet rich plasma (20 min, 2800 g, RT), and resuspended in a small volume of PBS/0.1% NaN_3. To remove erythrocytes, the suspension was diluted with an equal volume of lysis buffer (0.826% NH_4Cl/0.1% $NaHCO_3$/0.0037% EDTA pH7.4), chilled on ice for 10 min, and then washed with PBS/0.1% NaN_3. The platelets were stored at 4°C at least one month and washed three times with PBS/0.1% NaN_3 before absorption. The average yield was: 1.5 ml platelet pellet per 500 ml whole EDTA blood.

- platelet absorption

The platelets were isolated from the respective tissue donors, or pooled from several animals each carrying the class I antigen that had to be removed from the sera. Equal volumes of serum and packed platelets were carefully mixed and incubated: 45 min at RT, and subsequently 45 min at 4°C. During incubation the mixture was gently shaken. There after, the platelets were removed by three subsequent centrifugations (15 min, 17440 g, 4°C), and the sera were stored at -80°C. The absorption procedure was repeated if necessary.

- lymphocyte isolation

Peripheral blood collected in lithium heparin (14 I.E./ml) was centrifuged (10 min, 1000 g, RT), the buffy coat layer was aspirated and diluted with an equal volume of PBS. Peripheral blood nuclear cells, here referred as lymphocytes, were purified by Ficoll-metrizoate (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) gradient centrifugation (20 min, 1000 g) and re-suspended in RPMI-1640. When used for absorption, the lymphocytes were washed twice in PBS/0.1% NaN_3.

- lymphocytes absorption

Packed lymphocytes were carefully mixed with serum at a concentration of 10^9 packed cells per ml. and incubated: 1 h at RT, and subsequently 1 h at 4°C. During incubation the mixture was gently shaken. After absorption, the lymphocytes were removed by centrifugation (10 min, 15800 g, 4°C) and the sera were stored at -80°C.

MAILA assay

- test procedure.

The assay was performed as described by Mueller-Eckhardt *et al.* (1989) with minor modifications. Flat-bottom microtiter strips (Greiner B.V., Alphen a/d Rijn, The Netherlands) were prepared with 100 μl rabbit anti-mouse IgG_{2a} (Serotec code no: SERT 104, 3.1 μg/ml in 50 mM $CaCO_3$) per well. The test procedure was performed with fresh lymphocytes ($3x10^6$ cell/ml PBS). HRPO-labelled mouse anti-bovine IgG_1 and IgG_2 mixed 1:1, was used as conjugate. To block aspecific binding of this conjugate to the rabbit anti-mouse IgG_{2a} on the plate, the plates were incubated 15 min with wash buffer containing 5% mouse serum prior adding the conjugate. After incubation of 2 h at 4°C the wells were washed four times and 100 μl of substrate (OPD/UPO mixture, Organon Teknika B.V., Boxtel, the Netherlands) was added. After 10 min the colour reaction was stopped by adding 50 μl 4N H_2SO_4. The optical density was read at 492 nm and 620 nm in a titertek photometer. The test results are expressed by an optical density (OD) ratio, which is calculated by division of the OD value of a sample by the OD of the negative control. An OD ratio of $\geq$ 2.0 was considered as a positive reaction. Washbuffer was used as negative test control.

- monoclonal antibodies (mAb)

In the MAILA five mouse anti class II mAb were used for immobilization of the bovine class II molecules. The first mAb, IL-A21 recognises DRB3 gene products and is also applied for biochemical definition of DRB3 polymorphism. IL-A21 was

obtained from mice immunised with bovine lymphocytes (Teale, ILRAD, Nairobi, personal communication). The other four mAb, TH12B, H42A, TH22A5 and TH81A, were obtained from mice immunized with lymphocytes from multiple species in effort to elicit antibodies to highly conserved antigenic determinants (Davis *et al.* 1987). mAb TH12B is derived from the same cell-line as TH12A and is probably directed against bovine DR molecules. mAb H42A is directed against bovine DR molecules, and possibly other class II gene products, e.g. human DP. mAb TH22A5 and TH81A are DQ specific, but also show crossreactivity with DR (Bissumbhar *et al.* 1994). All five mAb have the IgG_{2a} isotype.

RESULTS

Production of Ds03 related alloantisera

The immunizations, which were focused on three 1D-IEF defined subtypes of Ds03, namely Ds03 DRBF03, Ds03 DRBF07 and Ds03 DRBF08, resulted in seven positive alloantisera (Table 2). Screening for class I and class II specific antibodies was performed on a cell panel of 25 animals, which included the skindonors. All sera showed anti lymphocyte reactivity. In the sera W1881, W0531, W0533 and W2268, this could be clearly attributed to the class I specificity of the donor. The other three sera showed multispecific or unclear reactivity patterns (data not shown). Based on the outcome of the class I screening the sera were absorbed with platelets to remove the anti T-cell reactivity and subsequently screened in the TCF for anti B- and T-cell reactivity (Table 3). Each serum appeared to have a distinctive anti B-cell reaction pattern. Three Ds03 related subgroups could be distinguished, which were locally designated as: Ds10, Ds11 and Ds15 (Table 2). In serum W0533 class I reactivity (A16) was not removed completely by one platelet absorption. The sera W0533, W2245, W2268 and W2283 reacted with all Ds03 positive cells if used undiluted, whereas W2268 and W2283 also reacted with Ds01-09 and Ds01-12 positive cells (data not shown).

Lymphocyte absorption

Heterogeneity of five Ds03 related alloantisera was studied by lymphocyte absorption. To discriminate between anti Ds03 and subtypic reactivity, Ds03-11 DRBF07 (A), Ds03-10 DRBF03 (B), and Ds03-15 DRBF08 (C) positive lymphocytes

Table 2. Result of the *immunization for production of Ds03 related alloantisera: the operational serum* class I activity before platelet absorption and class II activity after platelet absorption. Difference in class I and class II antigens between donor and recipient is also given.

serum	class I incompatibility	class I activity before absorption		class II incompatibility		class II activity after absorption	
ID	serology	titer	spec.	serology	1D-IEF	titer	spec.
W1881	A05, w44, P01@	1:4	P01	Ds03, Ds15	DRBF08	neat	Ds15
W0467	A14, A08	>1:32	pos.	Ds03, Ds11	DRBF07	1:4	Ds11
W0531	A16	1:16	A16	Ds11	DRBF07	neat	Ds11
W0533	A16	1:64	A16	Ds03, Ds11		1:8	Ds11
W2245	A16	>1:32	A16*	Ds03, Ds11	DRBF07	1:64	Ds11
W2268	A32	1:128	A32	Ds03, Ds11	DRBF07	1:16	Ds11
W2283	A19, A06	>1:32	A06	Ds03, Ds10	DRBF03	1:4	Ds03
E6689*	--	NT	--	NT	DRBF03	1:8	Ds03
B0597*	--	NT	--	Ds03	DRBF03	1:16	Ds03

* Immunization performed by Williams *et al.* 1991 (E) and Davies & Antczak 1991 (B)
NT Not tested
pos. Unclear reaction pattern
@ P01 is a local class I specificity (P01 = w44 + A05 + P03)

were selected (Table 4) for absorption. Ds01-12 DRBF06 (D) and Ds01-09 DRBF07 (E) positive lymphocytes were used to study the extra reactivity detected in some of the Ds03 related sera. The absorbed sera were screened in the TCF assay on a cell panel of 40 animals. Reaction on 24 representative animals is shown in Table 4. Serum W2268, W2283 and B0579 contained extra non Ds03 related reactivity. This reactivity was removed easily by all five lymphocyte samples, irrespective of their class II typing.

Absorption of the platelet absorbed fraction of serum W2245 (anti Ds11) with Ds03-10 (B), Ds03-15 (C), Ds01-12 (D) and Ds01-09 (E) positive cells had no noticeable effect. Absorption with Ds03-11 (A) positive cells removed all reactivity of W2245.

Serum W2268 contained broad (Ds03 related) reactivity that was removed by all five cells. The remaining activity of the absorption was comparable to that of serum W2245, although some what less specific. Absorption with Ds03-15 (C) positive cells removed part of the extra Ds03 related reactivity, leaving the reactivity towards Ds03-11 positive cells.

Table 3. Reaction pattern of the seven alloantisera before and after a platelet absorption. The standard microcytotoxicity assay was used for screening of the unabsorbed sera (L), whereas the TCF was used for screening of the platelet absorbed sera (B,T).

PANEL CELLS						W1881			W0467			W0531			W0533			W2245			W2268			W2283		
							abs			abs			abs			abs			abs			abs			abs	
CELL ID	CII A	CIII Ds	/	CII A	CIII Ds	L 23	B 01	T 01	L 45	B 23	T 23	L 23	B 01	T 01	L 45	B 34	T 34	L 56	B 56	T 56	L 45	B 45	T 45	L 45	B 23	T 23
0515	14	03-11	/	19	01	--	--	--	88	84	--	64	88	--	88	86	--	66	88	--	64	86	--	88	--	--
0513	14	03-11	/	17	02	--	--	--	86	88	--	64	88	--	88	88	--	86	88	--	44	88	--	88	--	--
0557	14	03-11	/	32	##	--	--	--	88	88	--	4-	8-	--	--	88	--	86	88	4-	88	86	--	4-	--	--
1335	16	03-11	/	32	02	--	--	--	42	86	--	88	8-	--	88	88	64	88	88	--	88	86	--	44	--	--
0475	16	03-11	/	15	01-09	--	--	--	88	88	--	88	88	--	88	88	88	88	88	--	44	8-	--	66	--	--
0458	16	03-11	/	19	03-10	--	--	--	88	....	....	88	....	....	88	....	....	86	88	--	64	86	--	88	8-	--
0516	11	03-10	/	19	01-12	--	--	--	86	--	--	--	--	--	64	--	--	--	4-	--	4-	4-	--	88	88	--
2042	11	03-10	/	03	01-05	84	--	--	6-	--	--	--	--	--	88	--	--	44	--	--	6-	--	--	66	8-	--
2301	19	03-10	/	31	01-05	--	--	--	88	--	--	--	--	--	88	--	--	4-	--	--	--	--	--	88	8-	--
1980	19	03-10	/	14	02	--	--	--	88	--	--	--	--	--	44	....	--	6-	--	--	6-	--	--	88	88	--
0503	19	03-10	/	11	03-10	--	--	--	86	--	--	--	--	--	86	--	--	64	--	--	--	4-	--	88	8-	--
0531	19	03-10	/	20	01	--	--	--	86	--	--	--	--	--	84	--	--	--	--	--	4-	--	--	88	6-	--
2005	05	03-15	/	44	02	88	8-	--	--	--	--	4-	--	--	86	--	--	4-	--	--	88	--	--	66	--	--
1703	05	03-15	/	44	02	88	8-	--	--	--	--	--	--	--	88	--	--	6-	--	--	88	--	--	4-	--	--
1457	05	03-15	/	44	02	....	8-	--	....	--	--	....	--	--	....	--	--	....	--	--	....	--	--	....	--	--
0533	15	01-09	/	14	13	--	--	--	86	--	--	--	--	--	--	--	--	88	--	--	4-	--	--	--	--	--
0461	18	01-09	/	19	01-06	--	--	--	88	--	--	--	--	--	4-	--	--	--	--	--	44	--	--	88	--	--
0549	18	01-09	/	12	01-08	--	--	--	86	--	--	--	--	--	88	--	--	--	--	--	--	6-	--	88	--	--
2251	20	01-12	/	17	02	--	--	--	88	--	--	--	--	--	66	--	--	--	--	--	--	--	--	88	....	....
0467	20	01-12	/	32	##	--	--	-	--	--	--	--	--	--	--	--	--	--	--	--	88	....	....	--	....	....
1983	14	02	/	14	02	--	--	-	88	--	--	--	--	--	--	--	--	6-	--	--	4-	--	--	--	--	--
1743	14	02	/	10	16-04	-	--	-	88	--	--	--	--	--	8-	--	--	--	--	--	86	--	--	--	--	--
1867	14	02	/	10	16-04	6-	--	-	88	--	--	--	--	--	86	--	--	4-	--	--	64	--	--	--	--	--
1102	16	02	/	38	01-06	-	--	-	86	--	--	88	--	--	88	88	88	88	--	--	6-	--	--	88	--	--
0530	32	02	/	20	01	-	--	-	--	--	--	--	--	--	--	--	--	--	--	--	88	--	--	--	--	--

: sero-type not detected with the available class II sera

L = all lymphocytes, B = B-lymphocytes, T = T-lymphocytes, abs = platelet absorbed

Used dilutions: 0 (neat), 1 (1:2), 2 (1:4), 3 (1:8), 4 (1:16), 5 (1:32), 6 (1:64)

Strenght of cytotoxicity: - = 0 - 20% cell death; 4 = 20 - 40% cell death; 6 = 40 - 80% cell death; 8 = 80 - 100% cell death; .... = not typed

Table 4. Reaction pattern of five sera before (O) and after lymphocyte absorption (A, B, C, D and E) on B-lymphocytes of 24 representative animals tested in the TCF. Lymphocytes used for absorption were isolated from animals 0513 (A), 2390 (B), 2323 (C), 2297 (D) and 2346 (E).

PANEL CELLS @				SERUM W2245*						SERUM W2268						SERUM W2283						SERUM E6689						SERUM B0579					
					lymp. absorbed						lymp. absorbed						lymp. absorbed						lymp. absorbed						lymp. absorbed				
CELL ID	CIII Ds	/	CIII Ds	O	A	B	C	D	E	O	A	B	C	D	E	O	A	B	C	D	E	O	A	B	C	D	E	O	A	B	C	D	E
				67	56	56	56	56	56	45	45	45	45	45	45	34	23	23	23	23	23	56	34	34	34	34	34	45	01	01	01	01	01
A-0513	03-11	/	02	88	–	88	88	88	88	88	–	88	88	88	88	88	–	–	–	66	–	88	–	–	–	88	88	88	–	4-	–	88	88
2322	03-11	/	13	88	–	88	8-	88	88	88	–	88	88	88	88	88	–	–	–	64	–	88	–	–	–	88	88	86	–	–	–	86	6-
2358	03-11	/	13	88	–	88	86	88	88	88	–	88	88	88	88	88	–	–	–	86	6-	88	–	–	–	88	88	8-	–	–	–	88	86
2331	03-11	/	16-04	88	–	88	88	88	88	88	88	88	88	88	88	88	–	–	–	8-	68	88	–	–	–	88	88	88	–	–	–	88	88
B-2390	03-10	/	03-10	–	–	–	–	–	–	88	–	–	–	6-	–	88	88	–	88	88	88	88	88	–	8-	88	88	88	8-	–	6-	86	8-
2360	03-10	/	03-10	–	–	–	–	–	–	88	–	–	–	8-	–	.8	88	–	.8	88	..	88	88	–	86	88	88	88	–	–	–	88	88
2042	03-10	/	01-05	–	–	–	–	–	–	88	–	–	–	–	–	88	88	–	88	88	88	88	88	–	86	88	88	88	6-	–	–	88	88
2360	03-10	/	13	–	–	–	–	–	–	88	–	–	–	–	–	88	88	–	88	88	88	88	88	–	84	88	88	86	–	–	–	6-	6-
C-2323	03-15	/	01-14	–	–	–	–	–	–	88	86	86	–	88	8-	48	88	–	–	88	88	88	86	–	–	88	88	88	–	–	–	88	88
2330	03-15	/	13	–	–	–	–	–	–	88	–	6-	–	.8	88	88	–	–	–	88	8-	88	88	–	–	88	88	88	–	–	–	88	88
2366	03-15	/	01-12	–	–	–	–	–	–	88	–	–	–	–	–	88	..	–	–	..	..	88	88	–	–	88	88	88	–	–	–	86	6-
2083	03-15	/	16-04	–	–	–	–	–	–	88	–	–	–	–	–	88	4-	–	–	88	86	88	84	–	–	88	88	88	–	–	–	88	86
2135	03-15	/	02	–	–	–	–	–	–	88	–	–	–	6-	–	88	4-	–	–	88	84	88	8-	–	–	88	88	88	–	–	–	88	84
2389	03-15	/	13	–	–	–	–	–	–	88	–	–	–	–	–	88	6-	–	–	66	66	88	86	–	–	88	88	88	–	–	–	86	8-
D-2297	01-12	/	01-12	–	–	–	–	–	–	66	–	–	–	–	–	8-	–	–	–	–	–	–	–	–	–	–	4-	6-	–	–	–	–	–
2399	01-12	/	01-12	–	–	–	–	–	–	88	–	–	–	–	–	88	–	–	–	–	–	8-	–	–	–	–	8-	88	–	–	–	–	–
2148	01-12	/	01-12	–	–	–	–	–	–	8-	–	–	–	–	–	8-	–	–	–	–	–	–	–	–	–	–	–	6-	–	–	–	–	–
2243	01-12	/	01-04	–	–	–	–	–	–	..	..	..	–	–	–	8-	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
E-2346	01-09	/	01-09	–	–	–	–	–	–	88	–	–	–	–	–	84	–	–	–	–	–	–	–	–	–	–	–	64	–	–	–	–	–
1992	01-09	/	02	–	–	–	–	–	–	64	–	–	–	–	–	82	–	–	–	–	–	–	–	–	–	–	–	6-	–	–	–	–	–
0533	01-09	/	13	–	–	–	–	–	–	86	–	–	–	6-	6-	8-	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1983	02	/	02	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2337	13	/	13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2122	01-08	/	01-06	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

@ for explanation see footnote table 3

* serum W2245 has been absorbed with platelets prior the lymphocyte absorption

Absorptions of sera W2283 and E6689 resulted in similar panel reactivity. Ds01-12 (D) and Ds01-09 (E) positive cells had no effect on the Ds03 related reactivity, whereas Ds03-11 (A) positive cells removed anti Ds03 and Ds11 but left Ds10 and Ds15 reactivity. Absorption with Ds03-15 (C) removed Ds03, Ds11 and Ds15 reactivity. Ds03-10 (B) positive cells completely removed the reactivity of these two sera.

Absorption of serum B0579 with Ds01-12 (D) and Ds01-09 (E) positive cells had no noticeable effect on the Ds03 related reactivity. Weak reactivity towards some Ds03-10 positive cells was detected after Ds03-11 (A) and Ds03-15 (C) absorption. Absorption with Ds03-10 (B) positive cells removed all the anti B cell reactivity of B0579. These results show that all five sera contain antibodies towards antigenic determinants associated with specificity Ds03. In addition the sera contain antibodies distinct from the Ds03 specific antibodies. Serum W2245 and W2268 contain antibodies towards antigenic determinants associated with specificity Ds11, and serum W2283 and E6689 contain antibodies towards antigenic determinants associated with specificity Ds10. Possibly serum B0579 also contains antibodies for the Ds10 associated determinant. The reactivity of serum W2283 and E6689 towards Ds03-10 and Ds03-15 positive cells indicates presence of Ds10+15 determinant shared by these haplotypes.

MAILA

For further characterization of the Ds03 sera, the MAILA assay was applied. Class II (DR and DQ) molecules, were immobilized using five mAb. With the DR specific mAb IL-A21 distinct reaction patterns on the immobilized molecules could be distinguished (Table 5). Similar patterns were observed using TH12B and H42A confirming their anti DR specificity. Serum W2245 contained antibodies against DR molecules of Ds03-11 positive cells as well as antibodies against the DR molecules of the other Ds03 positive cells tested. Serum W2268 only contained antibodies against DR molecules of Ds03-11 positive cells, and serum W2283 contained antibodies against DR molecules of Ds03-10 positive cells. Although only a limited number of animals was tested with the DQ specific mAb, a striking difference in reactivity of TH22A5 and TH81A was observed. DQ specific mAb TH81A reveals a Ds03 associated reactivity in all three sera. In serum W2268 and W2283 also extra reactivity towards the DQ molecules of the Ds01-09 and Ds01-12 positive cells was detected. With mAb TH22A5 only the Ds01 related reactivity was detected.

Table 5. MAILA analysis of three Ds03 related sera expressed as OD ratio. OD ratios ≥ 2.0 are shown.

PANEL CELLS			W2245					W2268					W2283				
CELL ID	CIII Ds	/ CIII Ds	IL-A21	TH 12B	H 42A	TH 22A5	TH 81A	IL-A21	TH 12B	H 42A	TH 22A5	TH 81A	IL-A21	TH 12B	H 42A	TH 22A5	TH 81A
0557	03-11	/ ##	3.8	6.5	3.8	---	3.6	3.0	5.3	2.4	---	5.4	.....	.....	.....	.....	.....
0475	03-11	/ 01-09	2.0	2.1	---	---	---	2.0	3.5	---	4.1	3.6	---	---	---	3.6	5.8
1335	03-11	/ 02	6.3	10.2	6.5	2.5	3.9	2.2	4.4	2.5	---	5.8	---	---	---	---	4.9
0513	03-11	/ 02	2.4	6.1	3.1	---	---	---	3.2	2.0	---	4.1	.....	.....	.....	.....	.....
0458	03-11	/ 03-10	7.6	15.2	9.6	---	9.3	2.9	6.7	6.7	---	10.5	8.0	7.7	4.5	---	12.8
1980	03-10	/ 02	2.0	3.2	4.2	---	2.8	---	---	---	---	4.0	4.3	4.4	2.9	2.3	4.6
0516	03-10	/ 01	---	2.1	---	---	---	---	---	---	---	2.1	2.9	2.6	2.3	---	2.5
2301	03-10	/ 01-05	---	2.2	2.9	---	2.3	---	---	---	---	2.8	---	2.9	2.1	---	2.9
2042	03-10	/ 01-05	2.0	3.1	4.8	2.1	4.1	---	---	---	---	5.4	.....	.....	.....	.....	.....
2005	03-15	/ 02	---	2.5	6.8	---	3.6	---	---	2.2	---	5.4	.....	.....	.....	.....	.....
0461	01-09	/ 01	.....	.....	.....	.....	.....	---	---	---	3.7	2.9	.....	.....	.....	.....	.....
0549	01-09	/ 01-08	.....	.....	.....	.....	.....	---	2.0	---	3.5	3.6	---	---	---	4.0	3.6
2251	01-12	/ 02	.....	.....	.....	.....	.....	---	---	---	5.4	2.3	---	---	---	4.9	2.4
0467	01-12	/ ##	.....	.....	.....	.....	.....	---	---	---	3.0	---	.....	.....	.....	.....	.....
1743	02	/ 16-04	---	---	---	---	---	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1102	02	/ 01-06	---	---	---	---	---	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1983	02	/ 02	.....	.....	.....	.....	.....	---	---	---	---	---	---	---	---	---	---

Sero-type not detected with the available class II sera
--- OD ratio < 2.0
..... Not done

DISCUSSION

Alloimmunizations focused on three 1D-IEF defined subtypes: Ds03 DRBF03, Ds03 DRBF07 and Ds03 DRBF08 were performed, to produce Ds03 subtypic alloantisera. In these immunizations donor and recipient were not matched for class I because platelet absorption appeared functional to remove class I reactivity in the antisera. Williams *et al.* (1991), observed that biochemical defined disparity could evolve specific alloantibodies. In the study described here, three immunizations across slightly different class II haplotypes (Ds– DRBF07 (W0531), Ds03 DRBF– (W0533) and Ds03 DRBF07 (W2245)), resulted in sera with the same Ds11 specificity (Tables 2 and 3), whereas screening of the undiluted serum fractions also revealed Ds03 specific antibodies in serum W2245 and W0533. In animal 0533 identity for DRBF07 did not prevent immunization against Ds11 showing that in production of specific class II alloantisera in cattle class II serotyping is at least as important as the 1D-IEF typing for DRB3. It also illustrates that the DRBF07 type associated with Ds01 is immunologically different, from DRBF07 associated with Ds03, which was confirmed during the Fifth International BoLA Workshop (Davies *et al.* 1994a).

The immunization of animal 0531, without Ds03 disparity resulted in a Ds11 specific serum without Ds03 specificity, indicating that DRBF07 associated determinants are seen as antigens that are distinctly different from Ds03. Cross-absorption of the Ds03 related sera with lymphocytes confirmed that Ds03 and Ds11 were distinct specificities.

The absorption study also revealed the presence of other distinct antibody populations, which were associated with the serological defined specificities Ds10 and Ds15. One of these antibody populations indicated the presence of a Ds10+15 determinant. DQ-RFLP analysis of these cells revealed association of the serotype Ds10 with DQ haplotype 1A and Ds15 with DQ haplotype 7, respectively (data not shown). Sigurdardóttir *et al.* (1992), suggested close relationship between these two DQB alleles. Therefore the Ds10+15 determinant may be DQ encoded.

Differentiation between DR and DQ directed antibodies was not possible by the lymphocyte absorption study. Therefore the MAILA assay was used to immobilize DR and DQ molecules separately. Interpretation of the MAILA data, is influenced by two factors: the specificity of the mAb that is used to immobilize the molecule, and the specificity of the antibodies reacting with the immobilized molecules. For DQ immobilization two mAb were used. Specific DQ reactivity of these mAb was suggested by Davis *et al.* (1987), and is confirmed by recent biochemical studies,

which however also revealed cross-reactivity with DR molecules (Bissumbhar *et al.* 1994). However due to the striking differences in reactivity of TH22A5 and TH81A as compared to the DR specific mAb, specific immobilization of DQ molecules was assumed.

Table 5 shows absence of reaction of Ds03 sera towards the TH22A5 immobilized molecules of Ds03 positive cells, which is contradicted by the reaction patterns seen in TH81A. This could be caused by two reasons, first: absence of specific Ds03 associated DQ antibodies, and second: absence of reaction of the mAb with certain DQ molecules. The latter appears to be the case, TH22A5 does recognise a determinant on DQ molecules, but apparently this determinant is not present on Ds03 positive molecules. Non reactivity of TH22A5 was confirmed in complement dependent cytotoxicity assays on Ds03 homozygous animals (data not shown). Therefore this mAb is not informative for the Ds03 DQ associated haplotypes in this study.

The MAILA-analysis was thus interpretated as follows. Serum W2245 contained DR specific, cytotoxic Ds11 antibodies, and DR specific non-cytotoxic Ds10 (+ Ds03) antibodies. Serum W2268 contained DR specific, cytotoxic Ds11 antibodies, whereas serum W2283 contained DR specific, cytotoxic Ds10 antibodies. Both W2268 and W2283 contained DQ specific, cytotoxic broadly reactive antibodies. This antibody population includes the extra reactivity with the Ds01-12 and Ds01-09 positive cells. As all three sera contained Ds03 specific reactivity in the TCF (if used undiluted), the DQ specific antibodies associated with Ds03 are interpreted as DQ specific, cytotoxic Ds03 antibodies.

Immunization against the same Ds03 DRBF07 haplotype resulted in similar, but slightly different antibodies (W2245 and W2268). The Ds01-12 haplotype of recipient 2245 apparently prevented the production of broadly reactive DQ antibodies which were observed in serum W2268.

The immunogenic composition of class II haplotypes where several DR specificities share the same serological DQ specificity is strikingly similar to the first observations in human (Duquesnoy et *al.* 1979). Like in man, a mixture of DR and DQ antibodies in the alloantisera complicated discrimination between DR and DQ specificities.

In conclusion, alloantisera that are subtypic to Ds03 can be produced. Absorption studies revealed that these sera contained several antibody populations. Some of these were reactive with distinct antigenic determinants, and some reacted with determinants shared by different haplotypes. Using the MAILA, part of the complex

reactivity pattern in the sera could be unravelled. Evidence is provided for antigenic determinants on the bovine DQ molecules that are associated with the Ds03 specificity. Determinants defined as Ds11, Ds10 and possibly also Ds15, are most likely located on the DR molecules and encoded by different alleles of the DRB3 gene.

Unravelling the heterogeneity of the Ds03 alloantisera not only provides insight in their complexity, but also give insight in the composition of the immunogenic determinants of BoLA-Dw03 associated haplotypes.

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Chapter 5

BIOCHEMICAL CHARACTERIZATION OF BOVINE MHC DQ ALLELIC VARIANTS BY ONE-DIMENSIONAL ISOELECTRIC FOCUSING

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ABSTRACT

Previous studies on expressed bovine MHC class II polymorphism using one-dimensional isoelectric focusing (1D-IEF) enabled the identification of at least 12 allelic variants of the DRB3 gene. So far, only limited data was available on the expression of other class II genes. The present study involved biochemical analysis of bovine MHC class II molecules using a set of monoclonal antibodies presupposed to be bovine DR and DQ reactive. After essential modification of the standard electrophoresis conditions used for 1D-IEF typing of bovine DR products, biochemical polymorphism was observed for non-DR molecules, revealing polymorphic sets of basic and acidic focusing bands. Because of the extensive DNA polymorphism described for bovine DQA and DQB genes, and the apparent similarity with the focusing pattern of human DQ products, these molecules were considered to be the bovine DQ homologues. The definition of the DQ associated banding patterns was enabled by the usage of two half-sib sire families. Four different DQA-like patterns and nine DQB-like patterns were detected. Segregation of the DQ types was supported by serological class I and class II typing. These results show that it is now possible to discriminate between expressed bovine DR and DQ polymorphism.

Keywords: BoLA, bovine MHC, class II, DQ products, isoelectric focusing

INTRODUCTION

To study the expressed polymorphism of bovine MHC class II genes, both one-dimensional isoelectric focusing (1D-IEF) and serotyping are currently the techniques of choice. In cattle, class II serology has only recently become fully operational. At present, with the class II specific sera available, only haplotype variation can be detected. Discrimination between expression of DR or DQ products is not yet possible. In this respect, the 1D-IEF technique appears more useful (Davies *et al.* 1994a).

The 1D-IEF studies on class II polymorphism in cattle have been particularly informative in the sense that they provided the initial framework for class II typing at the product level. Expression of a polymorphic DR-α/ß heterodimer was demonstrated in family segregation studies (Joosten et *al.* 1989). Comparison of DR typing at the DNA level by restriction fragment length polymorphism (RFLP) and typing at the product level by 1D-IEF confirmed that the class II polymorphism detected by these two methods were significantly correlated (Joosten *et al.* 1990). Subsequent studies have revealed that the DRB3 gene is apparently the only DRB gene expressed on peripheral blood mononuclear cells (Burke *et al.* 1991).

Expression of polymorphic DQ-α/ß heterodimers has not yet been demonstrated. Genomic studies of bovine MHC class II genes, however, have revealed considerable DQ polymorphism. The presence of 20 DQA RFLP types and 17 DQB RFLP types was demonstrated by Sigurdardóttir (Sigurdardóttir *et al.* 1988, 1991). Besides, two distinct types of DQA genes were reported, which are represented by the genomic clones W1 (Van der Poel *et al.* 1990) and A5 (Sigurdardóttir *et al.* 1991). Also, the number of DQA and DQB genes was found to vary between the haplotypes.

Although an extensive DQ polymorphism was found on the DNA level, the question remained to what extent this DQ polymorphism, is expressed at the cell surface.

Currently, the most straightforward way to study the expression of DQ polymorphism in cattle is the biochemical characterization of DQA and DQB allelic variants. Therefore, in this study the 1D-IEF protocol used for typing of expressed DR products was modified to enable the detection of products other than DR. SDS-PAGE and two-dimensional gel electrophoresis were used to confirm the class II nature of the precipitated products. Two of the four monoclonal antibodies (mAb) used, precipitated class II molecules that are likely the expressed products of the DQ region. These putative DQA and DQB allelic variants were correlated with class II serotypes, and with class II DRB3 haplotypes as defined by 1D-IEF.

MATERIALS AND METHODS

Animals

Two paternal half-sib families of Holstein-Friesian animals, each comprised of four dam (D) - offspring (O) combinations (a total of 16 animals), were used. In addition 15 Holstein-Friesian animals from an outbred population were selected for this study.

Monoclonal antibodies

The monoclonal antibodies (mAb) used for immunoprecipitation were: TH14B, TH22A5, TH81A and IL-A21. The mAb TH14B, TH22A5 and TH81A were derived from mice, immunized with a mixture of lymphocytes from multiple species (horse, rabbit, rat, dog, goat cattle and man), and thought to recognize the putative DR and DQ products (Davis *et al.* 1987). The mAb IL-A21 (a gift from Dr. A.J. Teale) was derived from mice immunized with bovine lymphocytes. This mAb was used for the 1D-IEF typing of bovine DR products in the Fifth International BoLA workshop (Davies *et al.* 1994a).

Immunoprecipitation and one-dimensional isoelectric focusing

Immunoprecipitates of class II bovine lymphocyte antigens (BoLA) were analyzed by 1D-IEF as described previously (Joosten *et al.* 1989, Davies *et al.* 1994a) with some modifications. Briefly, membrane proteins of ^{35}S methionine labelled peripheral blood mononuclear cells, separated on a Ficoll-Isopaque gradient, were extracted using a 1% NP40 lysis buffer. After preclearing, the class II molecules were precipitated using a set of class II reactive mAb. Neuraminidase digested samples were analyzed by 1D-IEF.

To enable IEF analysis of DQ-like molecules, the IEF protocol used for DR typing had to be modified. The following modifications were applied: 1. an additional preclearing step of 30 minutes using normal rabbit serum (NRS) and 70μl of a 10% Staph. aureus suspension (Behring Diagnostics) was introduced; 2. the composition of the gels was changed to 4.5% acrylamide:bisacrylamide (30:0.8 stock, Millipore), 9M urea, 2% NP-40, 4.5% (w/v) ampholines (4% pH 5-7, 1% pH 3.5-10, 0.4% pH 7-9; LKB); 3. the concentration of ampholine in the sample buffer was changed to 4.8% (v/v) ampholine pH 3.5-10 (+) (Millipore); 4. in addition to 2-mercapto-ethanol, di-thio-threitol (DTT) (to a final concentration of 1.5%) was added to the sample buffer, to enhance the breakage of sulphate bridges; and 5. the gels were run for 18 hours at a constant current of 15 mA and 50 Watt, with the voltage limited to 800 V. Gels were fluorographed using DMSO-PPO and were autoradiographed on Kodak X-AR film for 1-2 weeks. Class II DQ analysis by 1D-IEF was performed using the mAb TH22A5 and TH81A. For each mAb 3 μl of a stock solution of 1 mg/ml in PBS were used. The modified protocol was also used for the detection of DR polymorphism. DR products were precipitated with 3 μl TH14B. The DRB-region focusing (DRBF) nomenclature presented at the Fifth International BoLA Workshop was used (Davies *et al.* 1994a). As a negative control we used an isotype matched mAb recognizing

uteroglobulin. This mAb lacked the ability to precipitate any radioactive labelled material from cell lysates.

Two-dimensional gel electrophoresis

Gel electrophoresis was performed as described by McMillan *et al.* (McMillan *et al.* 1987) with the following modifications: a) ampholines pH 5-7 and 3.5-10 were added to a final concentration of 4% and 1%, respectively, b) an overlay solution was not used, c) the IEF gel was run for 14 hours at 300 V followed by one hour at 800V.

SDS-Polyacrylamide gel electrophoresis

For the SDS-PAGE analysis, precipitates prepared without neuraminidase treatment, were run overnight in a 12.5% polyacrylamide gel at 20 mA and a maximum of 120 V. Gels were fluorographed as described above for IEF.

Serological typing

For serological class I typing the complement mediated double-stain fluorescence lymphocyte microcytotoxicity assay was used (Bruning *et al.* 1982). For class II typing the two colour fluorescence technique (TCF) was used (Van Rood *et al.* 1976). The nomenclature used for the BoLA class I antigens (A) is that presented in the report of the Fifth International BoLA Workshop (Davies *et al.* 1994b). For the class II antigens local designation (Ds) was used (Nilsson *et al.* 1994a).

RESULTS

To establish the expressed polymorphism of bovine MHC class II DQ genes three mAb, TH14B, TH22A5 and TH81A together with a reference mAb IL-A21 (Davies *et al.* 1994a), were used in 1D-IEF analysis. SDS-PAGE analysis and two-dimensional gel electrophoresis were employed to confirm the anti-MHC class II DR and/or DQ-like reactivity of the different mAb. Figure 1 shows the SDS-PAGE results from samples of a single heterozygous animal, revealing MHC class II molecules with molecular weights (Mr) ranging from 27kD - 35kD. Notably, distinct molecular species were precipitated by the different mAb. In comparison to the apparent broad recognition pattern of IL-A21, which appeared to include all of the products precipitated by the other mAb, the recognition pattern of the other three mAb appeared far more restricted. In the Fifth International BoLA Workshop IL-A21 was defined as primarily

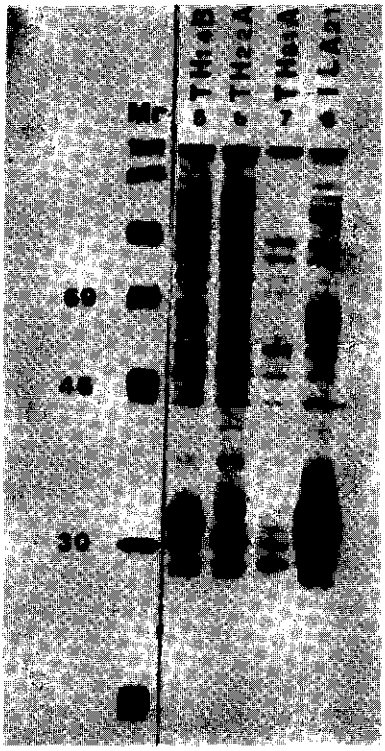

Figure 1. Class II BoLA antigens precipitated from cell lysates from non-stimulated peripheral blood mononuclear cells (PBMC) were analyzed by 12.5% SDS-PAGE. Lane 1 and 4: antigens precipitated by mAb TH14B and IL-A21. Lane 2 and 3: antigens precipitated by mAb TH22A5 and TH81A. The precipitated samples were not digested by neuraminidase. Mr = relative mass marker protein.

DRB3 reactive (Davies *et al.* 1994a), but it was also clear that precipitations with this mAb revealed minor bands in addition to the major DRB associated doublets. Based on the observation that TH14B, TH22A5 and TH81A each recognized a restricted but discrete subset of class II molecules, these mAb were selected for further 1D-IEF analysis with the aim to detect expressed polymorphism of class II loci besides DR. For 1D-IEF analysis, two paternal half-sib families including, eight dams and their offspring, were used to enable the definition of maternal and paternal haplotype associated banding patterns. The definition of the haplotypes was supported by serological class I and class II typing. Table 1 shows the typing results of both half-sib families. The 1D-IEF results are exemplified in Figures 2, 3 and 5, where the segregation of banding patterns within one of the two families is shown.

Table 1. Typing results from two half-sib families: BoLA class I (A) and class II (Ds) as defined by serology, and 1D-IEF types (DRBF, DQAF, DQBF and DwF). Homozygosity was confirmed by typing of related animals.

	class I (A)	class II (Ds)	TH14B (DRBF)	TH22A5 (DQAF)	TH22A5 (DQBF)	TH81A (DwF)
Sire 59						
D1 1959	11 / 05	03-10 / 03-15	03 / 08	- / 03	- / 06	09 / 06
O1 2324	20 / 05	01-12 / 03-15	06 / 08	01 / 03	04 / 06	04 / 06
D2 2116	10 / 13	16-04 / 03-15	09 / 11	04 / 03	07 / 06	- / 06
O2 2357	12 / 13	03-10 / 03-15	03 / 11	- / 03	- / 06	09 / 06
D3 2161	11 / 11	NT / NT	05 / 03	01 / -	03 / -	03 / 09
O3 2408	20 / 11	01-12 / 03-10	06 / 03	01 / -	04 / -	04 / 09
D4 2170	20 / 19	01-12 / 03-10	06 / 03	01 / -	04 / -	04 / 09
O4 2412	12 / 19	03-10 / 03-10	03 / 03	- / -	- / -	09 / 09
Sire 62						
D5 1982	19 / 05	03-10 / 03-15	03 / 08	- / 03	- / 06	09 / 06
O5 2330	14 / 05	13 / 03-15	01 / 08	- / 03	01 / 06	01 / 06
D6 1846	14 / 12	13 / 01-08	01 / 05	- / 01	01 / 02	01 / 02
O6 2339	19 / 12	03-10 / 01-08	03 / 05	- / 01	- / 02	09 / 02
D7 1981	31 / 05	03-15 / 03-15	08 / 08	03 / (03)	06 / 08	06 / 08
O7 2378	19 / 05	03-10 / 03-15	03 / 08	ND / ND	ND / 08	09 / 08
D8 2007	15 / 20	01-09 / 01-12	07 / 06	02 / 01	05 / 04	05 / 04
O8 2395	14 / 20	13 / 01-12	01 / 06	- / 01	01 / 04	01 / 04

NT Not tested
ND Not defined
- No product

Using the BoLA Workshop protocol, 1D-IEF analysis using TH14B exclusively revealed the characteristic DRB doublets as previously observed with IL-A21, indicating the DR specificity of TH14B (data not shown). However, under the workshop conditions isoelectric focusing of molecules precipitated by TH22A5 or TH81A did not meet with the same success, since the molecules did not migrate through the gel or the 1D-IEF resulted in a dirty smear. Systematically applied changes in the protocol finally improved the resolution. Details are listed in the Materials and Methods section. With the modified protocol the DR associated banding patterns using TH14B, were not influenced. Segregation of the DRB associated banding patterns can be seen in Figure 2, in which 1D-IEF analysis with

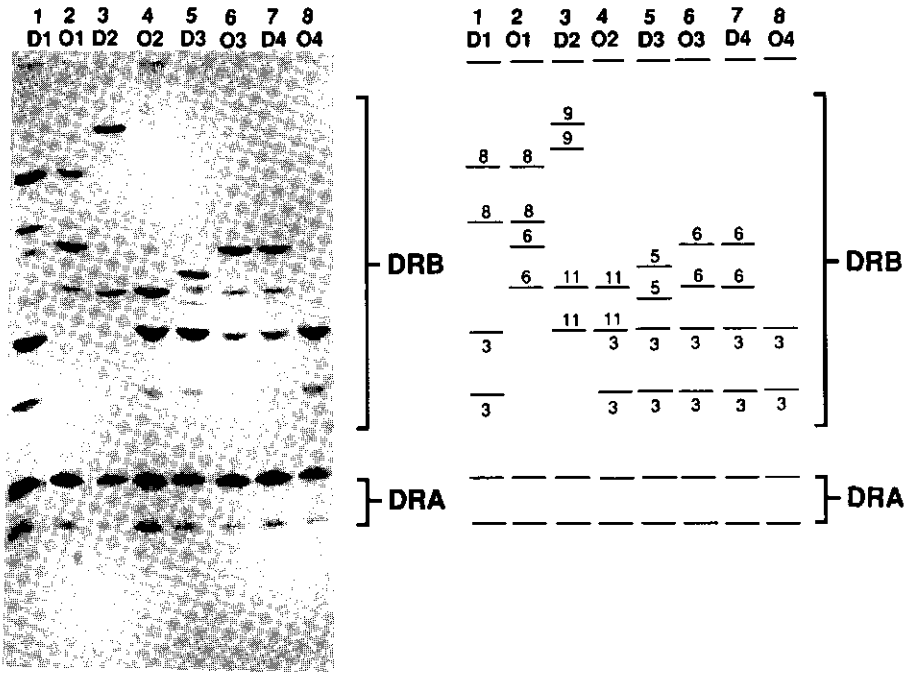

Figure 2. 1D-IEF analysis of BoLA DR antigens precipitated from cell lysates from non-stimulated peripheral blood mononuclear cells (PBMC) by the mAb TH14B, in a half sib family of eight animals, comprised of four dams and their offspring. The left part of the figure represents the autoradiograph, the right part shows an interpretative drawing of the haplotypes (DRBF). Dams are represented in the lanes next to their offspring (D = Dam, O = Offspring).

TH14B of four dam - offspring combinations is shown. In the sixteen animals used in this study eight DRBF types were detected (Tables 1 and 2).

The banding patterns obtained with TH22A5 and TH81A differed considerably from those obtained with TH14B, suggesting that these mAb predominantly recognize products of loci other than DR. On the basis of similarity with the HLA DQ 1D-IEF defined banding patterns (Rodriguez de Cordoba *et al.* 1987), the TH22A5 precipitated bands were putatively designated as DQ-like products: the products were represented by two polymorphic sets of bands, one focusing in the basic area of the gel (DQB-like) and one focusing in the acidic region of the gel (DQA-like). For each

animal, the TH22A5 precipitated bands that focused in exactly the same position as the TH14B precipitated bands were considered to be DRB products. The degree of apparent cross-reactivity of the mAb with the various DR products is indicated in Table 2.

Table 2. BoLA class II DR-DQ haplotypes as defined by serology and 1D-IEF.

SERII (Ds)	TH14B (DRBF)	TH22A5 (DQAF)	TH22A5 (DQBF)	TH81A (DwF)	TH22A5 DR prec.*	TH81A DR prec.*	DQA-genes**	Sire Hapl. (N) #
13	01	-	01	01	+	+	W1	2
03-10	03	-	-	09	+	+	W1	6
01-08	05	01	02	02	+	+	W1 + A5	1
NT	05	01	03	03	+	+	W1 + A5	1
01-12	06	01	04	04	+	+	W1 + A5	3
01-09	07	02	05	05	+	+	W1 + A5	1
03-15	08	03	06	06	+	+	W1 + A5	3
03-15	08	ND	08	08	+	+	W1 + A5	1
16-04	09	04	07	-	+/-	+/-	A5 + A5	1
03-15	11	03	06	06	+/-	+/-	W1 + A5	1
01-04##	04	01	07	-	+	+	A5 + A5	1
NT##	07	02	03	03	+	+	W1 + A5	1

NT Not tested
ND Not defined
\- No product
* Cross-reactivity of TH22A5 and TH81A with DR products
** All animals were typed for DQA by RFLP (unpublished data), and the DQ-gene type was inferred from Sigurdardóttir *et al.* (6)
\# Number of sire haplotypes
\## Additional haplotypes defined in the population

Figure 3 shows the 1D-IEF analysis of TH22A5 precipitates from the same four dam - offspring combinations also shown in Figure 2. The DQ-like types were defined based on the pI values and the segregation of the banding patterns after subtraction of the DRB associated bands and as yet unidentified bands. Eight distinct DQB-like banding patterns (DQBF01-08) and four distinct DQA-like banding patterns (DQAF01-04) were defined (Table 2).

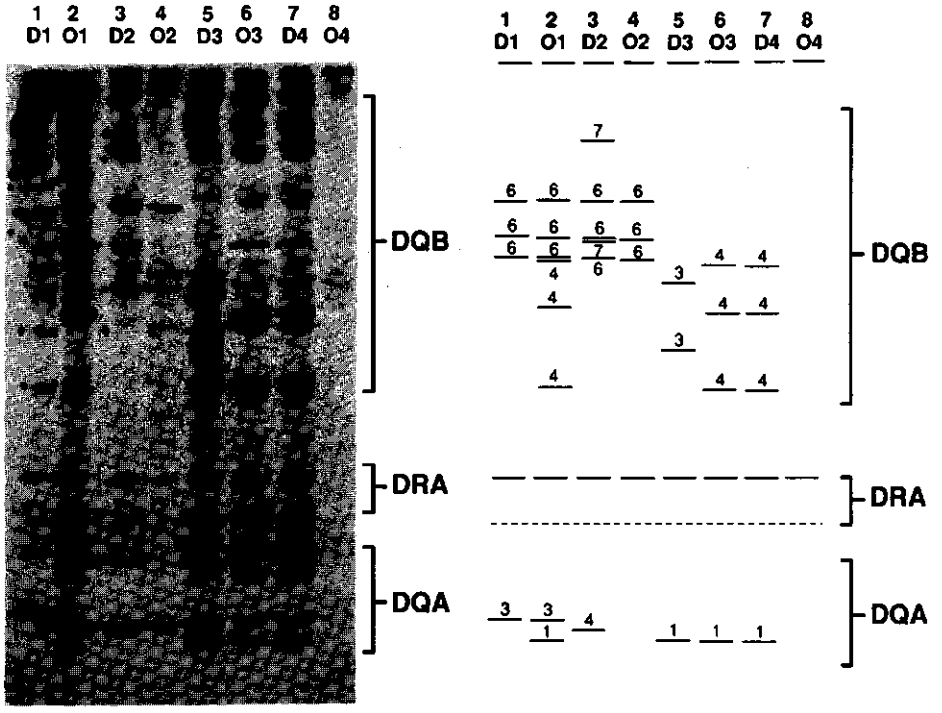

Figure 3. 1D-IEF analysis of BoLA class II antigens precipitated from cell lysates from non-stimulated peripheral blood mononuclear cells (PBMC) by the mAb TH22A5, in a half sib family of eight animals, comprised of four dams and their offspring. The left part of the figure represents the autoradiograph, the right part shows an interpretative drawing of the putative DQ types. Dams are represented in the lanes next to their offspring (D = Dam, O = Offspring). On the autoradiograph bands in addition to those shown in the interpretative drawing can be seen. Most of these extra bands are thought to be DR associated bands (indicated by *), whereas some of the bands can as yet not be explained.

The MHC nature of these bands was confirmed by two-dimensional gel electrophoresis. As an example, samples from a single animal precipitated with TH14B and TH22A5 are shown in Figure 4.

Notably, TH22A5 did not precipitate any products from the haplotypes defined as Ds03-10 DRBF03. Furthermore, no precipitation of DQA-like products was seen in haplotypes defined as Ds13 or Ds03-15.

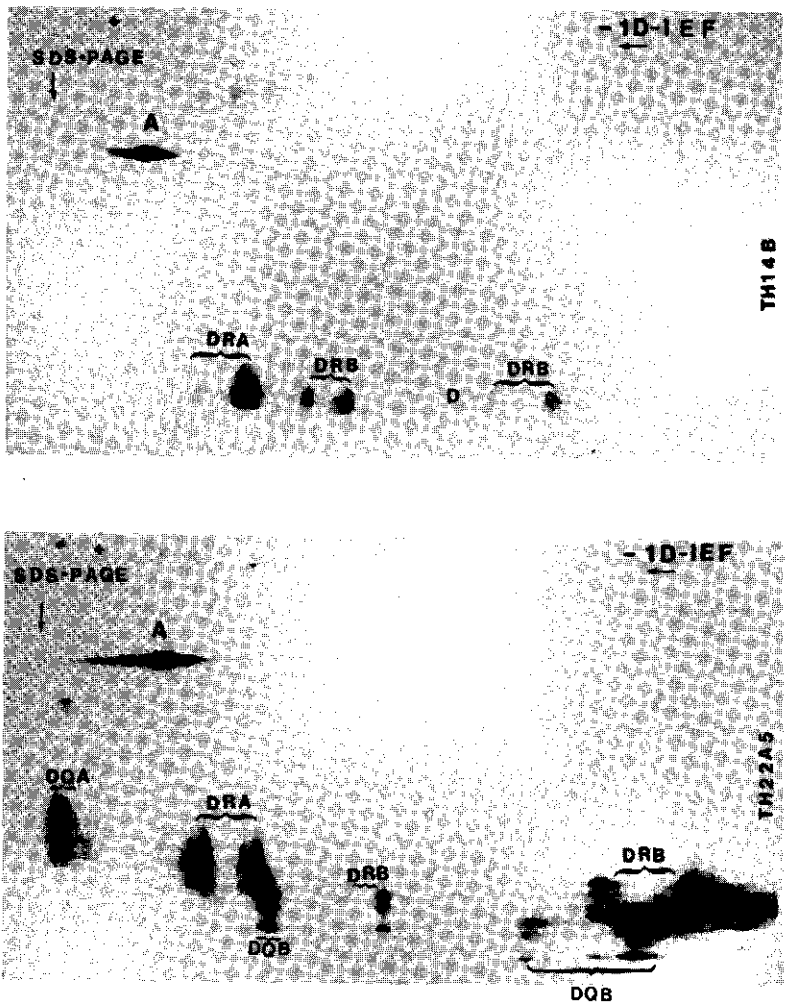

Figure 4. Two-dimensional gel electrophoresis of samples from a single heterozygous animal. The upper figure shows the results obtained with TH14B, in which the DRA and DRB associated spots are indicated. The lower figure shows the results obtained with TH22A5, in which the MHC nature of the 1D-IEF defined DQA and DQB-like associated banding patterns was confirmed. In addition, the co-precipitation of DR associated products is obvious.

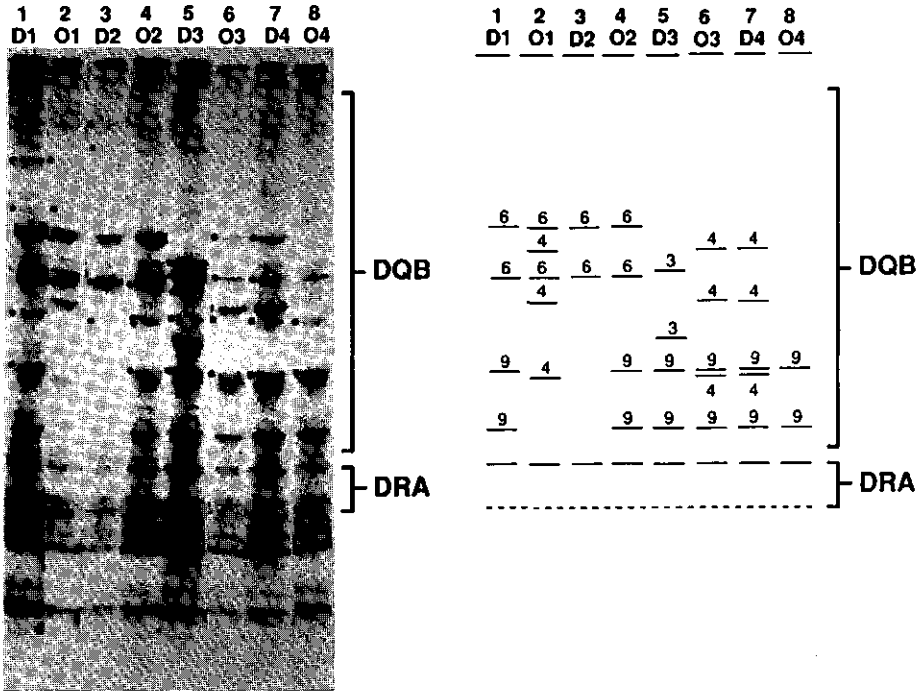

Figure 5. 1D-IEF analysis of BoLA class II antigens precipitated from cell lysates from non-stimulated peripheral blood mononuclear cells (PBMC) by the mAb TH81A, in a half sib family of eight animals, comprised of four dams and their offspring. The left part of the figure represents the autoradiograph, the right part shows an interpretative drawing of the putative DQ types. Dams are represented in the lanes next to their offspring (D = Dam, O = Offspring). On the autoradiograph bands in addition to those shown in the interpretative drawing can be seen. Most of these extra bands are considered to be DR bands (indicated by *), whereas some bands can as yet not be explained.

Figure 5 shows the 1D-IEF results obtained with TH81A. Although this mAb is said to have anti-DQ reactivity (Davis *et al.* 1987), it is surprising that in the acidic region of the gel, where the α-chains are expected to focus, no polymorphic bands are observed. Nonetheless, the banding patterns obtained with TH81A in the basic part of the gel were similar to those obtained with TH22A5 defining the putative DQB-like products. The TH81A bands are for the moment described by D-focusing (DwF), because the exact nature of these bands is not clear. The banding patterns obtained

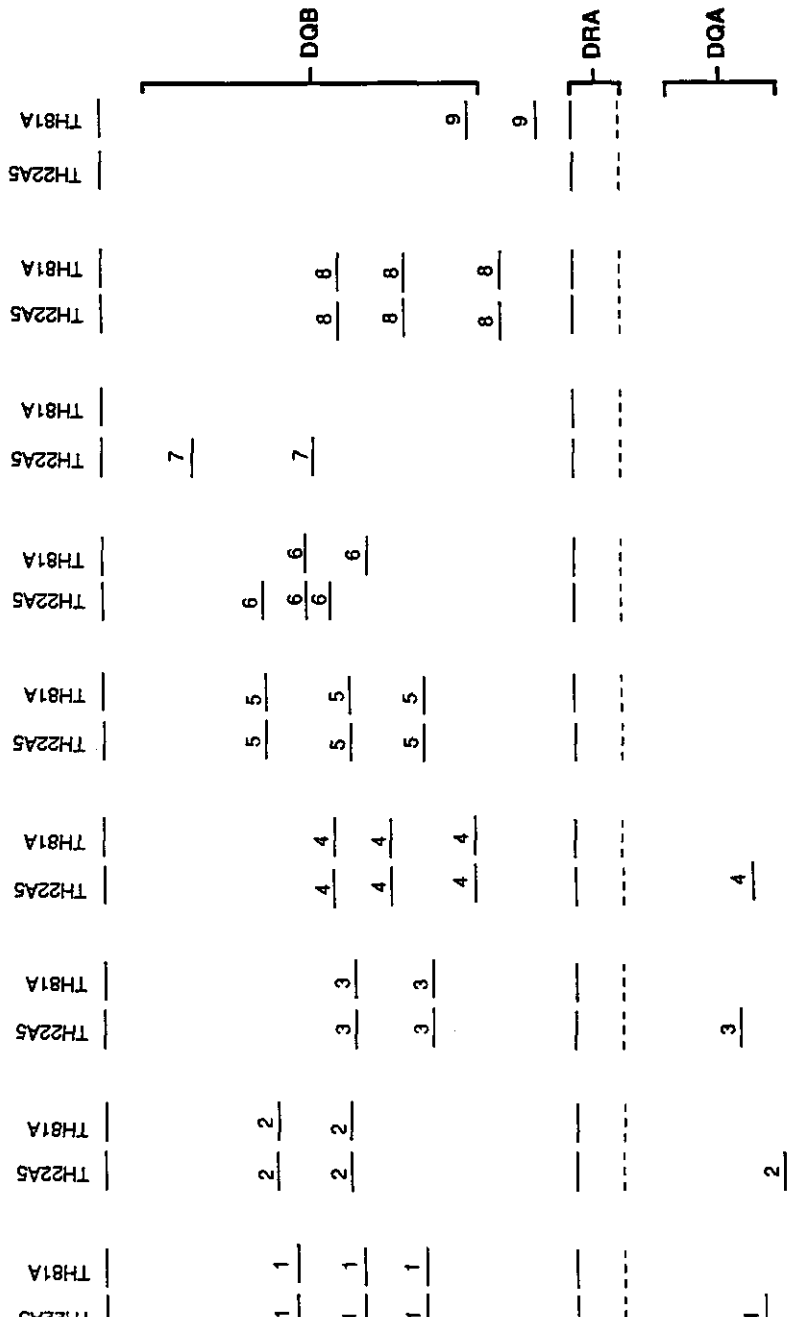

Figure 6. A schematic representation of the IEF banding patterns obtained with the two mAb TH22A5 (DQAF and DQBF types) and TH81A (DwF types) for each of the haplotypes defined. For DQA and DQB associations see Table 2.

by TH81A which were identical to the TH22A5 DQB-like patterns were DwF01-05 and DwF08. In a single case (DQBF06-DwF06) there was just one band in common between the TH22A5 and TH81A derived patterns (Figure 6). TH81A did not precipitate any products from the 'Ds16-04 DRBF09 DQBF07' haplotype. However, for the 'Ds03-10 DRBF03' haplotype, which was TH22A5 negative, a unique banding pattern was observed with TH81A, which was defined as DwF09.
We also typed non-related animals to confirm the typing data obtained with the two families. The 1D-IEF defined DQ-types detected in the two half-sib families were also detected in these animals. Moreover, two additional combinations of DQAF-DQBF versus DRBF types were detected (Table 2). These two extra haplotypes confirm the occurrence of a single DRBF type with two different DQF types, and also that of a single DQAF or DQBF type with two distinct DRBF types.

DISCUSSION

To date, the 1D-IEF defined polymorphism of the DRB3 gene is well established (Joosten *et al.* 1989, Joosten *et al.* 1990, Davies *et al.* 1994a). In contrast to DR, no data was available on the identification by IEF of products of other class II genes, although DNA studies on the bovine MHC class II region have indicated the presence of highly polymorphic DQA and DQB genes (Sigurdardóttir *et al.* 1988, 1991).

In the present study, 1D-IEF analysis was performed with three mAb, which were raised against a mixture of lymphocytes from multiple species, TH14B, TH22A5 and TH81A with the aim to detect conserved epitopes. TH14B appeared to be DR specific (Davis *et al.* 1987), whereas the latter two were predominantly reactive with the products of a second, non-DR, class II gene, likely DQ (Davis *et al.* 1987).

To enable the biochemical characterization of the molecules precipitated by TH22A5 and TH81A, a modification of the electrophoresis conditions as used for bovine DR typing, was essential. The poor migration into the standard 1D-IEF gels of molecules precipitated by TH22A5 and TH81A, indicated distinct structural properties of these molecules as compared to the DRA and DRB molecules. Interestingly, poor migration of class II molecules was also observed for HLA DPß chains (Baas *et al.* 1987), which was imputed to the poor solubility of these chains.

The banding patterns obtained with TH22A5 and TH81A were highly complex, comprising several major and numerous minor bands for each haplotype. In part this was caused by the apparent cross-reactivity of both mAb with DR molecules.

Sequential precipitations whereby lysates were depleted of DR molecules resulted in less complex banding patterns (data not shown). Unfortunately, for typing purposes, this proved far too laborious.

In addition to the DR products, TH22A5 revealed polymorphic non-DR associated putative α and ß chain products, the latter represented by three or even four bands. On the basis of the similarity with the focusing pattern of human DQ chains (Sigurdardóttir *et al.* 1991), and the DNA based polymorphism of bovine DQA and DQB genes (Sigurdardóttir *et al.* 1988, 1991), the DQ nature of the TH22A5 precipitated products seems for the moment sufficiently conclusive.

Only four different DQA patterns were detected. The different α chain variants seem to associate selectively with specific variants of the ß chain. Nevertheless, the DQAF01 is seen in combination with at least four DQBF types.

The reactivity pattern of TH81A showed some remarkable features. Although described as being anti-DQ (Davis *et al.* 1987), the IEF banding pattern obtained was not wholly identical to that of TH22A5. Most notably was the absence of acidic focusing polymorphic bands, whereas the set of more basic focusing bands appeared largely similar to the set obtained with TH22A5. This could suggest that TH81A recognizes non-complexed DQß chains, or that TH81A precipitates products of a third class II locus. The largely identical basic focusing pattern obtained for each haplotype would argue against the latter. The first option is complicated by the fact that TH81A is reactive in flow cytometry (data not shown) and MAILA (Monoclonal antibody-specific immobilization of lymphocyte antigen) analysis (Nilsson *et al.* 1994b) on the same haplotypes recognized here, suggesting the recognition of cell surface heterodimers. In the case of IEF the absence of a DQA associated band could be caused by hampered penetration of the DQα chain product into the 1D-IEF gel. At first this seems to be controversial, as with TH22A5 precipitation, penetration of DQα chains does occur. The explanation for this could be that in cattle several haplotypes comprise two distinct types of DQA genes, represented by the genomic clones W1 (Van der Poel *et al.* 1991) and A5 (Sigurdardóttir *et al.* 1991) (Table 2). Predicted on the basis of recent sequence data of DQA exon 2, the amino acid homology between genes of the two DQA types, W1 and A5, is only 60-70% (Van der Poel, personal communication). When DQA genes of both types are expressed, this is likely to result in the presence of two very distinct DQα chains.

It could well be that TH81A precipitates only DQ molecules complexed with a DQα chain encoded by alleles of one of the two DQA gene types: in this case the W1 type and not the A5 type (Table 2). Suggestive evidence for this reasoning is that

firstly, in the 1D-IEF analysis the haplotypes 'Ds16-04 DRBF09 DQAF04 DQBF07' and 'Ds01-04 DRBF04 DQAF01 DQBF07', were associated with a total absence of TH81A precipitated products. These two haplotypes carry the DQA*6A-DQB*6A and DQA*5-DQB*5 RFLP types, respectively (Van der Poel, unpublished results), and therefore only have DQA genes of the A5 type (Table 2). Secondly, we argue that in the W1 associated haplotypes only the DQß chains will be detected, assuming that the α-chain products encoded by genes of the W1 type do not penetrate into the gel due to distinct structural properties.

In contrast to TH81A, TH22A5 likely precipitates DQ heterodimers with DQα chains encoded by either W1 or A5 genes. Following our line of reasoning that DQA products associated with the W1 gene type do not easily penetrate into the gel, the acidic focusing bands detected in TH22A5 precipitation will have to be the products of the DQA A5 gene. This hypothesis is confirmed by the two haplotypes 'Ds13 DRBF01' and 'Ds03-10 DRBF03', which only have a single DQA W1 gene: no DQα bands were detected.

With TH22A5 there was total non-precipitation for cells with the haplotype Ds03-10. Selective absence of reactivity of TH22A5 was also seen in other studies. In the complement dependent cytotoxicity assay, as well as in the MAILA assay, TH22A5 appeared to be negative with cells homozygous for Ds03. This absence of TH22A5 reactivity towards Ds03 positive cells is intriguing. As the workshop cells positive for Ds03 all have the transmembrane RFLP pattern DQB-TM*45, and Ds03 positive cells manifest large similarity in the both DQA and DQB RFLP patterns, this non-reactivity might be due to variable affinity of TH22A5 for minor variation in the 'constant' regions of the α or ß chain molecules.

Comparison of the 1D-IEF defined DQ types with the class II haplotypes based on serology plus 1D-IEF DRB3 typing, revealed that one class II haplotype defined as 'Ds03-15 DRBF08' was associated with two different 1D-IEF defined DQ types (Table 2). This shows that the 1D-IEF DQ analysis enables a more extensive discrimination of the haplotypes defined by both serology and 1D-IEF DRB3 typing.

In conclusion, it has become possible to describe the expressed polymorphism of a second bovine class II allelic series, the putative bovine DQ products, using IEF. So far, only a restricted number of variants could be established. Based on the latest BoLA workshop results (Davies *et al.* 1994a), the number of variants is expected to be much higher. More extensive IEF analysis combined with both serology and DNA based techniques should be used to further unravel the class II polymorphism.

ACKNOWLEDGMENTS

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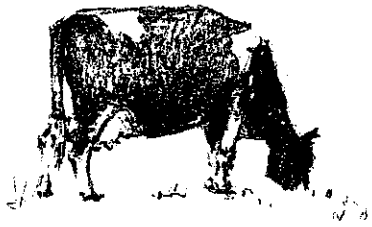

Chapter 6

CHARACTERIZATION AND FREQUENCY DISTRIBUTION OF BOVINE MHC HAPLOTYPES IN HOLSTEIN FRIESIANS

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CHARACTERIZATION AND FREQUENCY DISTRIBUTION OF BOVINE MHC HAPLOTYPES IN HOLSTEIN FRIESIANS.

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SUMMARY

In the present study, typing data on the bovine MHC polymorphism are presented. These data are gathered during a 5 year period on 686 Holstein Friesian cows, part of an experimental herd of the Wageningen Agricultural University. These animals were typed for MHC class I and class II using the following methods: class I and class II serology, 1D-isoelectric focusing for DRB3, typing for DQA and DQB by restriction fragment length polymorphism (RFLP) analysis and for DRB3, using polymerase chain reaction RFLP.

In addition to the Fifth International BoLA Workshop (1992), 13 class II haplotypes and 44 class I - class II combinations were newly defined.
Moreover, as the typing was performed over a 5 year period, we were able to compare class I and class II type distribution over consecutive generations. Considerable fluctuations in frequency distribution were observed, as illustrated with class I serotypes and the class II DRB3-PCR types present in the herd.

Keywords: BoLA, bovine MHC haplotypes, serology, 1D-isoelectric focusing, RFLP

INTRODUCTION

In the Fifth International Bovine Lymphocyte Antigen (BoLA) Workshop, the Major Histocompatibility Complex (MHC) of cattle was investigated utilizing multiple typing techniques. This approach appeared to be very helpful, especially in the description of class II polymorphism: 38 class IIa (DR-DQ) haplotypes could be defined (Davies *et al.* 1994a). Good definition of the MHC class II region is indispensable, especially in research on T-lymphocyte responses towards T-cell sites on synthetic peptides designed to be used as vaccine components (Glass *et al.* 1991, Van Lierop *et al.* 1992), but also in MHC disease association studies (Xu *et al.* 1993).

Apart from defining the extent of bovine MHC polymorphism, also the knowledge concerning the distribution of this polymorphism in a population is needed. In cattle, little is known about the distribution or breed specific occurrence of bovine MHC polymorphism. The only information available is dealing with class I (Oliver *et al.* 1981, Bull *et al.* 1989, Mejdell *et al.* 1994). The Fifth International BoLA Workshop (FIBW) was not informative in this matter, as the 62 animals typed were from nine different breeds.

Recently, we described seventeen serologically defined class II haplotypes, their association with biochemical defined DRB3 types and their linkage with the class I serotypes (Nilsson *et al.* 1994). In order to extend this class II definition, which was only based on detection of expressed polymorphism, the animals were retyped, using two DNA based techniques: restriction fragment length polymorphism (RFLP) analysis for DQA and DQB, and polymerase chain reaction RFLP (PCR-RFLP) for DRB3. Furthermore, we proceeded by class I (serology) and class II (DRB3-PCR RFLP) typing of their pedigree.

We here present an overview of all our typing data for bovine MHC polymorphism obtained in one herd in the past five years. As these data have been obtained during an extended period, we were able to study the segregation of MHC haplotypes over several generations. This allowed identification and confirmation of virtually all the maternal and paternal haplotypes. Furthermore, we give the frequencies of the class I serotypes and class II DRB3-PCR types present in this herd. The distribution of these frequencies over the subsequent generations will be discussed.

MATERIALS AND METHODS

Animals

In this study 686 female cattle were used. These animals were Holstein-Dutch-Friesian crosses referred to as Holstein Friesians (HF). The animals belong to a single herd and were housed at an experimental farm of the Wageningen Agricultural University (WAU). The WAU herd has been founded in 1971, and in 1982 crossbreeding with HF-sires was started. The mean HF percentage of the sires used between 1982 - 1986 was 66%, and from the sires used between 1987 - 1993 was 95%. The mean percentage of the sires used between 1971 and 1981 is estimated on 41%.

Since all animals belong to one farm population, most of them were related through sire or dam. In fact, 530 animals were members of 47 sire families with at least 5 offspring each, and in 263 instances both the mother and the daughter were typed.

MHC typing

Class I

The animals were typed for class I polymorphism by the complement mediated double-stain fluorescence lymphocyte microcytotoxicity assay as described by Bruning *et al.* (1982). With the set of alloantisera used the following 33 different class I types could be detected: A2, A3, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, w17, A18, A19, A20, A21, A22, A24, w28, w29, A30, A31, A32, A34, w38, w42, w44, w45, P3 and P4. The nomenclature used for the class I antigens (A,w) is the same as presented in the class I report of the FIBW (Davies *et al.* 1994b). P03 and P04 are two local class I types.

Class II

Class II typing was performed using four different methods.

1. Serotyping was performed by the two color fluorescence technique (TCF) as described by Van Rood *et al.* (1976) with minor modifications as described by Nilsson *et al.* 1994. For the serological defined class II haplotypes the local designation (Ds) was used.
2. Biochemical typing (immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF)) was performed as described by Joosten *et al.* (1989), and Davies *et al.* (1994a, 1994b). 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). The nomenclature (DRBF) presented in the class II report of the FIBW (Davies *et al.* 1994a) is used.
3. DRB3-PCR RFLP was performed essentially as described by Van Eijk *et al.* (1992) and their proposed nomenclature was used.
4. RFLP for DQA and DQB was performed as described by Sigurdardóttir *et al.* (1991). For DQA-DQB typing fragments containing the first and second domain (exon 2 and exon 3) of DQA and DQB genomic clones were used (clone DQA: W1, clone DQB: Y1, Groenen *et al.* 1990, Van der Poel *et al.* 1990). The sizes of the molecular weight fragments were adjusted on the basis of the BRL/Lifetechnologies 1kb ladder, and the nomenclature used for the DQA and DQB RFLP typing was based on the nomenclature presented in the report of the FIBW (Davies *et al.* 1994a).

For reason of clarity, a D-haplotype (DH) code comparable to those introduced in the class II report of the FIBW (Davies *et al.* 1994a) was assigned to each class II haplotype.

RESULTS

MHC typing

A total of 686 animals were typed for class I by serology, and twenty-four different class I types were detected. The class I types A2, A7, A21, A22, A24, w28, w29, w42 and w45, were not detected in the WAU herd. Part of the class I typed animals (n = 325) were typed for class II with all four typing methods. This resulted in the detection of 17 serological class II haplotypes, 10 DRBF types, 15 DRB3-PCR RFLP, 19 DQA patterns and 15 DQB patterns. One new DQA pattern combination was found in 19 animals. This pattern was defined as DQA*7G. The following fragments hybridized selectively with the DQA probe: Taq I exon 2: 8.4-7.6-1.7, Pvu II exon 2: 9.9-(7.2), Taq I exon 3: 8.4-3.8-2.9-1.3 and Pvu II exon 3: 9.9-6.3. The DQB pattern of these animals was identical to the DQB*7A pattern.

Based on the combination of the serological, biochemical and molecular typing results, 27 class II haplotypes could be defined (Table 1). Compared with the FIBW 13 of these 27 D-haplotypes were newly identified.

Definition of MHC haplotypes

In general a class I and class II type will segregate together, as class I and class II are closely linked (Usinger *et al.* 1981, Lindberg *et al.* 1988). In the WAU herd, 56 class I - II combinations could be defined. Compared with the FIBW 44 of the 56 detected class I - II combinations were newly defined (Table 2).

In the definition of the class I - II combinations, further referred to as BoLA-haplotypes, all available information was used, i.e. in the case of a known segregating paternal or maternal BoLA-haplotype, the complementary haplotype opposite of the segregating haplotype could be deduced and was consequently used too. Each animal was assigned a paternal and maternal BoLA-haplotype, using the known of the family structures. Subsequently, the BoLA-haplotypes of the sires were inferred from their offspring. These haplotypes were called 'sire-haplotypes'.

Since several generations of animals were typed, it was possible to create pedigree trees and follow the segregation of the MHC haplotypes over three to four

Table 1. Class II haplotypes (DH) based on class II serotyping (Ds), biochemical typing (DRB3 1D-IEF) and typing by RFLP (DQA + DQB RFLP, DRB3 PCR-RFLP), present in 168 'sire-haplotypes'. Per class II haplotype, the number of sire-haplotypes (N) is given.

Class II haplotype						
DH	Class II serology (Ds)	DRB 1D-IEF (DRBF)	DQA RFLP	DQB RFLP	DRB3 PCR-RFLP	N*
03A#	01-17-14-06	05	10	10	3	5
03C	01-17-14-06	05	12	12	3	1
07A#	02	02	2	2	7	23
08A#	01-12	06	12	12	8	20
10C#	01-17-08	06	11B	11A	10	1
11A#	13	01	3A	3A	11	11
11C#	01-17-14	06	9A	9A	11	1
12C	01-16-05	05	13B	13C	12	7
12D	01-16-05	06	13B	13C	12	1*
16A#	01-17-08	05	11A	11C	16	9
18A#	01-16-04-07	04	5	5	18	9
22B#	01-09	07	9B	9B	22	11
22F#	09	07	3A	4	22	1
22H	01-09	07	9B	9A	22	1
22I	01-09	07	11A	11A	22	3
22J	03	05	9B	9B	22	1*
22K	Blanco	05	3C	3C	22	2
23B	03	06	7G	7A	23	1*
23C	03-15	11	7C	7A	23	1*
23D	03-15	11	7D	7A	23	1
23E	03-15	11	7G	7A	23	3
24A#	03-10	03	1A	1	24	31
26A	16-04	09	6A	6A	26	6
27A#	03-11	07	1B	1	27	7
28A#	03-15	08	7A	7A	28	7
28B	03-11	08	1B	1	28	1*
32A	01-17-14-05	01	9A	9A	32	3

\# Class II haplotype also described by Davies *et al.* (1994a)

* All 'sire-haplotypes' were confirmed in at least three animals, except those with the astrix. For these haplotypes the definition is based on one sire-haplotype detected in only one or two animals.

Table 2. Combinations of the class I serotypes and class II haplotypes, present in 168 'sire-haplotypes'.

Class I serotype (A,w)	Class II haplotype (DH)*
A03	12C(1)
A05	28A(5), 28B(1)
A06	10C(1)
A09	03A(1)
A10	03A(3), 03C(1), 18A(7), 26A(4)
A11	12C(3), 12D(1), 16A(1), 24A#(13)
A12	16A#(6), 18A(1), 24A(2)
A13	23B(1), 23C(1), 23D (1), 23E(2), 26A(1)
A14	07A(6), 11A#(8), 12C(1), 27A(1)
A15	11A(1), 22B#(9), 22I(1), 22H(1)
A16	07A#(1), 16A(1), 27A#(6)
w17	07A(7)
A18	18A(1), 22B(2), 22I(2)
A19	11A(2), 22F#(1), 24A#(16)
A20	08A#(19), 23E(1)
A30	22J(1)
A31	11C(1), 12C(2), 28A#(1)
A32	07A(5), 22K(2), 26A(1)
A34	28A(1)
w38	03A(1), 07A(1)
w44	07A#(3), 08A(1)
P03@	32A(3)
P04@	16A(1)

@ P03 and P04 are two local class I types

\# Class I - class II combination also described by Davies *et al.* (1994a)

* Between brackets are the number of 'sire-haplotypes' positive for the class I - class II combination

generations. In this way the sire from whom the maternal haplotype originated could be traced back. Therefore the 'sire-haplotypes' were not only defined via paternal, but also confirmed via maternal routes.

A total of 234 'sire-haplotypes' were defined, however only 168 of them were based on all five typing methods used. The other 66 'sire-haplotypes' were based on class I serology combined with DRB3-PCR RFLP. The class I - II combinations, observed in the 168 completely typed 'sire-haplotypes' are given in Table 1 and Table 2, as well as the number of combinations.

Between 1982 and 1993, 79 sires have been used at the WAU. From the 168 completely typed 'sire haplotypes', 103 can be attributed to these 79 sires, the other 65 descended from the sires used before 1982. As consanguinity between sires is very common in dairy production, we analyzed these 79 sires used between 1982 and 1993 for this aspect. It turned out that several of the sires were related, they were sharing father, grandfather and/or great-grandfather. One sire (sire 94) was ancestor of 37 of the 79 sires. Table 3 illustrates, that confirmation of several of the 'sire-haplotypes' was also possible through consanguinity between the sire's.

Table 3. Example of the kinship of sires used in the WAU herd.

SIRE	SS	SSS	SSSS	HAPLOTYPES OF SIRE (A-DH)	(A-DH)
37	284	247	094*	A19-DH24A	A11-DH24A
19	247	094		A19-DH24A	A10-DH26A
51	287	094		A19-DH24A	A31-DH11C
62	293	094		A19-DH24A	A14-DH11A
80	303	094		A19-DH24X	A15-DH22X
70	307	290	094	A12-DH16X	A20-DH08X
75	306	290	094	A12-DH16X	A13-DH23X
16	276	094		A12-DH16A	A17-DH07A
22	276	094		A12-DH16A	A14-DH07A
50	290	094		A12-DH16A	A10-DH03C
52	290	094		A12-DH16A	A06-DH10C

SIRE Sire identification number
SS Father of the sire
SSS Father of SS
SSSS Father of SSS
X Not typed for class II DQA and DQB
* Inferred MHC haplotypes sire 094: A19-DH24A/A12-DH16A

In the group of 325 completely typed animals, we found 31 animals homozygous for class I, 35 animals homozygous for class II, and 18 animals homozygous for both class I and class II. Notwithstanding the relatedness between the sires used in the WAU herd, only in two cases the paternal and maternal haplotypes of these class I - II

homozygous animals could have descended from the same 'ancestor' sire. Furthermore, only one cross-over resulting in recombination between class I and class II was detected, and confirmed by segregation of the recombinant haplotype (Table 4). In five more instances we have strong indication for a cross-over event in the class I - class II region. In these cases however, the recombinant haplotypes were not confirmed in subsequent generations.

Table 4. Example of a cross-over event leading to recombination between the class I and class II region.

IDNR	GENOTYPE	IDNR	GENOTYPE
Sire 54	w44-07A/A20-08A		
D1 1737-13	A20-08A/A16-27A	D5 1481-06	w17-07A/A10-18A
O1 2193-54	w44-07A/A16-27A	O5 2206-54	w44*08A/A10-18A
		↓	
D2 1925-28	A10-03A/A11-24A	2551-74	A15-22X/w44*08X@
O2 2253-54	w44-07A/A10-03A		
D3 2071-33	A11-12C/A10-26A		
O3 2296-54	A20-08A/A10-26A		
D4 1861-29	A10-26A/A19-11A		
O4 2236-54	A20-08A/A19-11A		

IDNR Identity number
D Dam
O Offspring of sire 54
@ Class II typing only based on DRB3-PCR RFLP

Frequency

Table 5 and Table 6 summarize the frequency distributions as present in the cows of the WAU herd typed in the past five years. The frequency distribution of the class I serotypes was based on the 1372 haplotypes of the 686 class I typed animals (Table 5, first column). As mentioned above only 325 out of these 686 class I typed animals were also typed for class II. After definition of the 168 'sire haplotypes', the DRB3-PCR RFLP type of 308 animals not typed for class II could be inferred. The

Table 5. Frequency of the class I types present in 1372 class I haplotypes. To illustrate the changes in the distribution of the frequency, the frequencies of the class I types of three generations are given: 83 heifers (III, 166 haplotypes), their mothers (II, 166 haplotypes) and their grandmothers (I, 156 haplotypes).

Class I (A,w)	type ALL (n = 1372) %	I (n = 156) %	II (n = 166) %	III (n = 166) %
A03	0.1	0.6	0.0	0.0
A05	3.1	2.6	3.6	1.8
A06	0.7	1.3	1.2	1.2
A09	1.5	2.6	2.4	1.8
A10	8.0	12.2	4.2	0.6
A11	10.1	9.0	10.8	10.8
A12	6.2	2.6	7.2	12.0
A13	4.2	3.2	7.2	9.0
A14	10.1	11.5	11.4	5.4
A15	8.9	3.2	7.8	16.9
A16	6.6	4.5	3.0	1.8
w17	3.1	2.6	2.4	1.2
A18	2.1	3.2	1.2	0.6
A19	10.0	9.0	9.0	7.2
A20	13.6	9.6	17.5	16.3
A30	0.2	0.0	0.0	0.0
A31	4.0	3.2	4.8	6.6
A32	3.0	5.1	1.2	2.4
A34	0.2	0.0	0.0	0.0
w38	0.6	1.3	0.6	0.0
w44	2.0	2.6	0.6	4.2
P03*	1.0	1.3	1.2	0.0
P04*	0.8	0.0	0.0	0.0
NT	0.0	9.0	2.4	0.0

* P03 and P04 are two local class I types.
NT Not typed

frequency distribution of class II DRB3-PCR RFLP types therefore is based on the 1266 haplotypes of the 325 completely typed animals and 308 inferred DRB3-PCR RFLP types (Table 6, first column).

The distribution of the frequencies appeared to be dynamic. To illustrate this, the frequency distribution of the class I types (Table 5) and the class II DRB3-PCR RFLP types (Table 6) from 83 heifers (III) is given, next to that of their mothers (II) and grandmothers (I). The 83 heifers are daughters of 13 different sires.

Table 6. Frequency of the DRB3-PCR RFLP types likely to be present in 633 animals, of which 325 are typed by DRB3-PCR RFLP. For the remaining 308 animals the DRB3-PCR RFLP type was inferred. To illustrate the changes of the distribution of the frequency, the frequency of the DRB3 PCR-RFLP types of three generations are given: 83 heifers (III, 166 haplotypes), their mothers (II, 166 haplotypes) and their grandmothers (I, 156 haplotypes).

DRB3 PCR-RFLP type	ALL (n = 1266) %	I (n = 156) %	II (n = 166) %	III (n = 166) %
03	4.5	6.4	3.6	1.8
07	11.8	16.7	9.0	8.4
08	13.6	7.7	15.7	16.3
10	0.9	1.3	1.2	1.2
11	7.6	5.8	8.4	4.2
12	3.9	4.5	4.8	5.4
16	6.6	3.2	6.6	12.0
18	3.8	6.4	3.0	0.6
22	11.0	7.7	7.8	18.1
23	4.3	4.5	6.6	9.0
24	16.9	13.5	15.1	13.3
26	2.4	2.6	0.6	0.0
27	5.8	3.2	3.0	1.2
28	5.6	3.8	6.0	7.2
32	1.1	1.3	1.2	0.0
NT	0.0	11.5	7.2	0.0

NT Not typed

Table 5 shows that, more than 50% of the class I types is contributed by five different class I types (A11, A14, A15, A19 and A20), and more than 50% of the class II DRB3 types is contributed by four DRB3 types (DRB3*07, DRB3*08, DRB3*22 and DRB3*24), when all available data are considered. Four class I - DRB3 combinations were frequent in these data: A11-DRB3*24 (8%), A15-DRB3*22 (8%), A19-DRB3*24 (8%) and A20-DRB3*08 (13%) comprising 37% of the MHC haplotypes and 61% of the genotypes.

At a high frequency of the class I type A14 (Table 5) and the class II type DRB3*07 (Table 6) did not coincide of a high frequency of the BoLA haplotype A14-DRB3*07. This can be explained by the fact that A14 occurs with five different class II haplotypes, and DRB3*07 occurs with six different class I types.

In the 83 heifers (Table 5 and 6), more than 50% of the class I types can be described by just four types (A11, A12, A15, A20) (Table 5, fourth column), whereas almost 60% of the class II types can be contributed to four DRB3 types (DRB3*08, DRB3*16, DRB3*22) (Table 6, fourth column). The most frequent class I - DRB3 combinations in the heifers were: A12-DRB3*16 (11%), A15-DRB3*22 (16%) and A20-DRB3*08 (16%). These three combinations comprise 43% of the MHC haplotypes, and 72% of the genotypes.

DISCUSSION

MHC typing and definition

Comparison of expressed class II polymorphism detected serotyping and 1D-IEF (for DRB3), with class II polymorphism present at DNA level, detected by two DNA based typing methods (DRB3-PCR RFLP, and RFLP for DQA and DQB), showed that the expressed class II polymorphism was mostly associated with DRB3-PCR RFLP. This might be expected, since DRB3-PCR RFLP detects variation in the polymorphic exon 2 of the DRB3 gene. Thus, actually, the DRB3-PCR based data confirmed the class II typing at the product level. The number of haplotypes detected by the DRB3-PCR typing was lower than the number of combinations of class II serology and 1D-IEF for DRB3 (n = 22).

In contrast, the typing for DQ polymorphism using the RFLP method increased the number of class II haplotypes detected with the combination of class II serology and 1D-IEF for DRB3. In three D-haplotypes, DH03, DH22 and DH23, one combination of serology and 1D-IEF is associated with two or even three different DQ types. There seems to be no clear cut reason why there should be more DQ types associated with the DRB*03, DRB3*22, and DRB3*23 gene types. It may be that the detected polymorphism is not functional, as RFLP polymorphism might be due to nucleotide substitution in non-coding regions. Better definition of DQ may add to better haplotype definition. The relevance of this additional polymorphism is not clear and needs further study.

Four class I - II combinations were present at high frequency and present in more than 10 'sire-haplotypes': A11-DRB3*24, A15-DRB3*22, A19-DRB3*24 and A20-DRB3*08 (Table 2). In the 83 heifers one other combination was present at elevated frequency (A12-DRB3*22). These five haplotypes were also observed, in the FIBW, and two combinations (A11-DH24A and A20-DH08A) were also present at

higher frequency. The occurrence in several breeds of these five class I - II combinations, strongly indicates that they might be conserved haplotypes. A comparable phenomenon also occurs in man, where the term 'ancestral haplotype' has been used to describe conserved MHC haplotypes, which appear to be identical between unrelated individuals (Dawkins et *al.* 1983, Kay et *al.* 1988). Occurrence of combinations of alleles in a haplotype at frequency higher than expected are also called defined as supratypes (Dawkins et *al.* 1983), extended haplotypes (Awdeh et *al.* 1983), or preferential allelic association (Festenstein et *al.* 1986). Additional analysis of other loci e.g. CYP21, TNF (encoding cytochrome P-450 steroid 21-hydroxylase and tumor necrosis factor respectively), or loci in the class IIb region might be very useful, to further investigate the occurrence of conserved MHC haplotypes in cattle. Moreover, in MHC-disease association studies preferably class I typing should be combined with class II typing. The whole MHC haplotype should be considered, especially in cases where the disease predisposing genes may be located at relatively large distance from the MHC region, or is not known at all.

In our data, 18 animals homozygous for both class I and class II were found. In two cases there were indications that MHC homozygosity could be caused by inbreeding. The data are not conclusive and to reveal if the amount of detected homozygosity is lower than expected, pedigree analysis has to be performed. This would be in accordance with the hypothesis of overdominant selection, or heterozygote advantage, proposed to explain the high degree of MHC polymorphism (Doherty and Zinkernagel 1975, Klein and Figueroa 1986).

In six instances, indications for class I - II recombination events were found. Recently, the distance between the loci of BoLA-DRB3 and BoLA-A has been reported to be about 1cM (Van Eijk et *al.* 1994). The occurrence of recombinational events, six out of 686 animals, would be in accordance with the suggested distance.

Frequency

Frequency distribution based on related animals of one herd is of little value in view of population genetics. However the frequencies provided in Tables 5 and 6 give an indication on distribution of MHC polymorphism in a small population. The data were collected over a five years period, and it appeared that the gene pool altered drastically in this period. These changes directly reflect the genetic constitution of the sires used for breeding, which was supposed to be completely independent from MHC.

A similar dynamic distribution of frequency as shown in Table 5 and 6 was found in the 'sire-haplotypes', when the sires were devided in three groups (i.e. used between 1971 - 1982, 1982 - 1986 and 1986 - 1993, respectively). Considering the mean HF percentage in these three sire groups, 41%, 66% and 95%, respectively, the change in frequency distribution observed in the three generations may be caused by cross breeding between purebred HF-sires with the animals of the herd.

The data in Tables 5 and 6 demonstrate that in a period of two generations, frequency distribution can change considerably, e.g. a striking increase was detected for haplotype A20-DH08A, whereas haplotype A10-DH18A has almost disappeared. These extreme fluctuations seem to be limited to a few haplotypes. Surprisingly, one haplotype appeared to be present at high frequency in all three generations: A11-DH24A, moreover DH24A occurs relatively frequent in several different breeds. Coincidentally this haplotype is referred to in several studies on risk for mastitis (Oddgeirsson *et al.* 1988, Lundén *et al.* 1990, Weigel *et al.* 1990, Våge *et al.* 1992). In these studies this haplotype is suggested to be associated with enhanced susceptibility for subclinical mastitis, as well as decreased clinical mastitis. Maybe this haplotype contains genes that influence the immune response, and that are somehow also involved in selection.

Notwithstanding the considerable changes in frequency distribution in the three generations, more than 50% of the MHC polymorphism was covered by only a few haplotypes in each generation (Table 5, 6). Suppose these data were representative for the distribution of a large population of Holstein Friesian cows, such disbalance in distribution of MHC polymorphism might have severe implications when a negative effect, associated with one of the frequent haplotypes, is introduced in the population.

In this paper we have extended the characterization of the class II haplotypes with 13 newly defined D-haplotypes. Moreover, by presenting frequency distribution of MHC polymorphism, we also provide some indication how the bovine MHC polymorphism might evolve on population level.

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Chapter 7

THE SPECIFICITY OF ANTI-HLA CLASS II MONOCLONAL ANTIBODIES IN CATTLE

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ABSTRACT

In the Eleventh International HLA Histocompatibility Workshop numerous anti-HLA class II monoclonal antibodies (mAb) were tested. For several of the polymorphic mAb, the epitopes for binding have been mapped within the antigen-binding site of the class II molecules. Screening of the available bovine DRB3 and DQB exon 2 sequences revealed that some of the key amino acid (AA) motifs of these epitopes were present in cattle as well, and the question was raised, whether this sharing of key AA motifs might cause interspecies cross-reactivity.

Eight polymorphic anti-HLA class II mAb (seven anti-HLA DRB1 and one anti-HLA DQB) were selected for analysis of their reactivity with bovine lymphocytes. In addition, the monomorphic anti-HLA class II mAb, 7.5.10.1, was selected for analysis, as this mAb was described to detect class II polymorphism in cattle.
Flowcytometry and lymphocyte microcytotoxicity testing revealed that five of the polymorphic anti-HLA mAb were reactive with bovine lymphocytes. Furthermore, the anti-bovine reactivity of 7.5.10.1. was confirmed. These findings were supported by biochemical analysis.

The anti-bovine reaction of the anti-HLA mAb did not correspond with the expected reaction, which was based on the presence of the AA, postulated to be responsible for recognition. Therefore, we suggest that the patterns of reactivity of the anti-HLA mAb are not always determined by one epitope.

Keywords: anti-HLA class II mAb, bovine MHC, interspecies cross-reaction

INTRODUCTION

The class II region of the major histocompatibility complex (MHC) contains polymorphic genes, which encode the molecules that can bind antigenic peptides. In man, monoclonal antibodies (mAb) have proven to be useful tools in discrimination between the class II molecules, not only in serology, but also in structural,

biochemical and functional studies. A large number of anti-HLA class II mAb have been tested now, and for several mAb the aminoacid (AA) responsible for epitope recognition by the mAb (key AA) have been mapped within the antigen-binding site of the class II molecules. Definition of the key AA of the epitopes was based on a comparison of multiple sequence alignments with the reaction patterns of mAb on large panels of homozygous B-lymphoblastoid cell lines and transfectant cells expressing selected class II genes (Marsh *et al.* 1992).

Definition of bovine DRB3 exon 2 sequences (Sigurdardóttir *et al.* 1991) enabled comparison with the human DRB1 exon 2 sequences. This revealed an extensive sharing of polymorphic residues between man and cattle for DRB (Andersson *et al.* 1991). Screening of the bovine DRB3 and DQB exon 2 sequences, revealed that some of the AA motifs postulated to be responsible for epitope recognition of the anti-HLA mAb are present in cattle as well. This sharing of key AA motifs, implicates that possibly man and cattle share structural characters, that are recognised by anti-HLA mAb. Trans-species reactivity of anti-HLA class II mAb, based on the sharing of the AA motifs responsible for mAb binding between species, has been observed in chimpanzee. The cross-reacting anti-HLA mAb were subsequently used in characterization of chimpanzee class II antigens (Marsh *et al.* 1992). So, it is not illogical to assume that there might be cross-reactivity of anti-HLA mAb with bovine class II antigens.

Cross-reaction of anti-HLA mAb that are raised against human surface antigens, with cells from other species, such as horse, pig, cow, sheep has already been demonstrated for several mAb (Lunney *et al.* 1983, Aasted *et al.* 1988, Jacobsen *et al.* 1993). Reactivity of monomorphic anti-HLA class II mAb with bovine B lymphocytes have been reported by Spooner and Ferrone 1984, but no relationship between the reactivity pattern and any BoLA (class I) specificity could be found for these mAb, and the mAb reactivity was suggested to be towards public determinants on the class II molecules. Recently, a monomorphic anti-HLA class II mAb (7.5.10.1) has been reported, that displayed anti-bovine reactivity, and that might be associated with BoLA class II specificity (Nilsson *et al.* 1994).

In cattle, only monomorphic mAb are available to study the class II molecules. Two mAb, IL-A21 and TH14B are now generally used for biochemical characterization of DRB3 allelic variants (Bissumbhar *et al.* 1994, Davies *et al.* 1994a). Two other mAb, TH22A5 and TH81A enable biochemical characterization of DQA and DQB allelic variants. However, these two mAb also react with the products of DRB3, therefore the analysis of bovine DQ products is still very complex

(Bissumbhar *et al.* 1994). Analysis of the reactivity of anti-HLA mAb on bovine lymphocytes might provide additional information on the described reaction pattern of the anti-HLA mAb, and might reveal a mAb that can be used in the study on bovine class II polymorphism. Moreover, it can contribute to the definition of epitopes that are responsible for binding of the mAb especially for those of which the key AA is present in both man and cattle. Conservation of such epitopes may prove to be relevant in evolution, and learn us more concerning peptide binding and T-cell recognition.

In this study the reactivity of nine anti-HLA class II mAb on bovine lymphocytes was studied by using flowcytometry and a lymphocyte cytotoxicity assay. Subsequently, biochemical analysis was performed to confirm and further analyze the reaction of the mAb.

MATERIALS AND METHODS

Cells

In cattle there are no homozygous typing cells available. Therefore, peripheral blood mononuclear cells (PBMC), further referred to as lymphocytes, were used. The lymphocytes were obtained from Holstein-Dutch Friesian crosses, further referred to as Holstein Friesians (HF), that are part of an experimental herd of the Wageningen Agricultural University (WAU). Most of the animals of this herd are typed for class I and class II by the classical typing methods (see below), and their MHC haplotypes are confirmed through paternal and maternal ancestry. Hence, the MHC types of the animals used in this study, as well as the assignment of homozygosity for class I and class II is considered to be reliable.

MHC typing

Class I polymorphism was detected by serotyping, using the complement mediated double-stain fluorescence lymphocyte microcytotoxicity assay (Bruning *et al.* 1982). Class II polymorphism was detected by the following methods: serotyping by the two color fluorescence technique (TCF) (Van Rood *et al.* 1976), biochemical typing (immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF) for DRB3 (Joosten *et al.* 1989, Davies *et al.* 1994a) and restriction fragment length polymorphism (RFLP) for DQA and DQB exon 2 (Sigurdardóttir *et al.* 1991), and polymerase chain reaction (PCR) combined with RFLP for DRB3 exon 2 (Van Eijk *et*

al. 1992). The nomenclature used for the BoLA class I antigens (A,w), biochemical class II typing (DRBF), DQA-DQB RFLP and DRB3 PCR-RFLP typing is that presented in the class I and class II report of the Fifth International BoLA Workshop (FIBW) (Davies *et al.* 1994a,b, Van Eijk *et al* 1992). For the serological class II antigens local designation (Ds) was used (Nilsson *et al.* 1994).

Concluded from the combination of the serological, biochemical and molecular typing results for each class II haplotype a D-haplotype code (DH) was used, comparable to the one introduced in the class II report of the FIBW (Davies *et al.* 1994). In this study 18 class II haplotypes were involved (Table 1).

Table 1. Class II haplotypes (DH) involved in the analysis of HLA-mAb defined by on class II serotyping (Ds), biochemical typing (DRB3 1D-IEF) and typing by RFLP (DQA+DQB RFLP, DRB3 PCR-RFLP).

DH	Class II Serology (Ds)	DRB3 1D-IEF (DRBF)	DRB3 PCR-RFLP	DQA RFLP	DQB RFLP
07A	02	02	7	2	2
08A	01-12	06	8	12	12
11A	13	01	11	3A	3A
22B	01-09	07	22	9B	9B
23E	03-15	11	23	7G	7A
24A	03-10	03	24	1A	1
03A	01-17-14-06	05	3	10	10
11C	01-17-14	06	11	9A	9A
12C	01-16-05	05	12	13B	13C
16A	01-17-08	05	16	11A	11C
18A	01-16-04-07	04	18	5	5
22I	01-09	07	22	11A	11A
22H	01-09	07	22	9B	9A
26A	16-04	09	26	6A	6A
27A	03-11	07	27	1B	1
28A	03-15	08	28	7A	7A
28B	03-11	08	28	1B	1
32A	01-17-14-05	01	32	9A	9A

Based on the class II haplotype definition, for the 18 class II haplotypes, the AA sequences of DRB3 and DQB exon 2 were inferred from the 32 DRB3 (Sigurdardóttir *et al.* 1991, Ammer *et al.* 1992, Xu *et al.* 1993, Andersson, personal communication) and 22 DQB exon 2 sequences (Sigurdardóttir *et al.* 1992, Xu *et al.* 1991) published to date (Table 2). This inference is justified by the following arguments. First, in cattle, for the class II haplotypes defined sofar, identical similar DRB and DQB sequences were obtained in different breeds. Also, we assumed the class II haplotypes used in this study, to be conserved. Second, the DRB3-PCR RFLP typing method is as close as we can get to the original sequence, without having to sequence every animal. We checked the presence or absence of the relevant restriction sites for all haplotypes tested and further, we assumed the occurrence of nucleotide substitutions in between the restriction sites to be very limited.

Concerning the inference of the DQB sequences, the following should be noted. Bovine DQ polymorphism is considerably complex: two DQB loci, and four different DQB genes are described, and haplotypes contain one, or two DQB genes (Sigurdardóttir *et al.* 1992). Furthermore, no such accurate typing method as the DRB3-PCR RFLP is yet available for DRB typing, and inference of the DQ types is based on analysis by RFLP therefore. Therefore, the inference of the DQB sequences is not as reliable as for DRB3.

Monoclonal antibodies (mAb) (Table 3).

Eight polymorphic anti-HLA class II mAb (NDS40, JS1, 24C7, TAL8.1, TAL12.1, TAL14.1, TAL15.1, and TAL16.1) were used in this study. Selection was based on the presence or absence of the AA sequence homologues to the key AA responsible for epitope recognition, as predicted from 32 DRB3 exon 2 sequences and 22 DQB exon 2 sequences (Table 2). One monomorphic anti-HLA class II mAb (7.5.10.1), was included in this study as this mAb detects class II polymorphism in cattle. Four anti-BoLA class II mAb (TH14B, TH22A5, TH81A and IL-A21) were used as positive reference. One anti-HLA mAb (7.3.19.1), in previous studies found to be non-reactive with bovine lymphocytes, was used as negative reference.

The anti-HLA class II mAb were derived from mice immunised with human lymphocytes (Bodmer *et al.*1984, Koning *et al.* 1986, Sachs *et al.* 1986, Bodmer *et al.* 1989, Madrigal *et al.* 1989, Bunce *et al.* 1990, Cayrol *et al.* 1992, Sadler *et al.* 1993). The reagents used in the analysis were derived from the stock used in HLA-class II typing. The mAb TH14B, TH22A5 and TH81A, were derived from mice, immunized with a mixture of lymphocytes from multiple species (horse, rabbit, rat, dog, goat,

Table 2. The amino acid sequence of the bovine DRB3 and DQB first domains (exon 2). A *plus* (+) indicates positions in the antigen binding site according to the proposed model of the class II molecule (Brown et al. 1988). A *dash* (-) indicates identity to the HLA-DRB1*0101 and HLA-DQB1*0501 sequence. A *back slash* (\) in position 65 of DRB*7 indicates a deletion of one amino acid in this allele.

DRB sequences.

DRB3-PCR		10	20	30	40	50	60	70	80	90
		+| + + |	+ + |	++ |	|	+ |	+ + + + | + + |	
	HLA-DRB1*0101	RFLWQ	LKFECHFFNG	TERVRLLERC	IYNQEESVRF	DSDVGEYRAV	TELGRPDAEY	WNSQKDLLEQ	RRAAVDTYCR	HNYGVGESFT
07	BoLA-DRB3*2	H--EY	STS-------	-----F-D-Y	FH-G--F---	---W------	----------	----/EI--R	A---------	----GV----
08	BoLA-DRB3*12	H--EY	T-K-------	-----F-N-Y	FH-G--F---	---W------	----------	-----EI--R	A---------	----------
11	BoLA-DRB3*									
22	BoLA-DRB3*16	H--QY	H-G-------	-------D-H	F--G--Y---	---WD-F---	------S---	------F--R	---E---V--	-----V----
23	BoLA-DRB3**	H--EY	Y-R-----D-	-----F-D--	YT-G--T---	---W--F---	----------	------F--E	---E--RV--	----------
24	BoLA-DRB3*1	H--EY	S-S-------	-----F-D-Y	YT-G--T---	---W--F---	-----Q----	------F--E	K--E--RV--	----GM----
01	BoLA-DRB3*5a	H--EY	H-S-------	---L-Y-D-Y	F--G--Y---	---W------	--------K-	-----EI--R	K--N------	-----V----
02	BoLA-DRB3*13	H--EY	--S-------	-----F---Y	F--G--Y---	---W------	--------K-	---------R	K--N------	-----V----
03	BoLA-DRB3*10a	H--EY	S-S-------	-----F-D-Y	FH-G--Y---	---W------	----QRV---	C-----F--R	A---------	----------
03	BoLA-DRB3*10b	H--EY	Y-R-------	-----F-D-Y	FH-G--Y---	---W------	----QRV---	C-----F--R	A---------	----------
09	BoLA-DRB3*3	H--EY	C-S-------	-----F---S	F--G--F---	---W------	--------K-	----------	K--N------	----------
10	BoLA-DRB3*11	H--EY	T-K-------	-----F-D-Y	FH-G--F---	---W------	--------K-	------F--E	K---------	----------
11	BoLA-DRB3*9a	H--EY	S-S-------	-----F---S	F--G--N---	---W------	----------	-----EI--R	K--N--RV--	----------
12	BoLA-DRB3*17	H--EY	ATS-------	-----F-H-Y	F--G--YA--	---W--F---	------K---	-----RI--R	E--Y------	----GV----
13	BoLA-DRB3*4	H--EY	STS-------	-----F-D-Y	F--G--Y---	---W--FQ--	--------K-	------I--R	E---------	----GV----
15	BoLA-DRB3*20a	H--EY	C-R-------	-------D-Y	F--G--R---	---W--F---	------S---	------F---	----------	-----V----
15	BoLA-DRB3*20b	H--EY	C-R-------	-------D-Y	F--G--R---	---W--F---	----------	------F---	----------	----------
16	BoLA-DRB3*15	H--EY	STS-------	-----Y-D-Y	FH-G--F---	---W------	-----RV--Q	L-G---T--R	E--Y------	-----V----
18	BoLA-DRB3*18	H--EY	ATS-------	-----F-H-Y	FH-G--F---	---W--F---	------A--Q	------T--R	E--Y------	----GV----
20	BoLA-DRB3*23	H--EY	T-K-------	-------D-Y	YT-G--T---	---W--F---	-----Q---Q	------T--D	E---------	Y---------
21	BoLA-DRB3*8	H--EY	ATS-------	-----F-D-Y	FH-G--L---	---W--F---	------S-VH	L-----F--D	E--S------	-----V----
26	BoLA-DRB3*6a	H--EY	C-R-------	-------D--	FH-G--F---	---W--F---	-----RV--H	L-----HI--R	K--E---V--	----------
27	BoLA-DRB3*14	H--QY	H-G-------	-------D-H	F--G--F---	---WD-F---	------A--Q	------F---	K--E--RV--	----GV----
28	BoLA-DRB3*7	H--EY	C-R-------	-----F-D--	FH-G--F---	---W--F---	-----RV--Q	------F--E	---E--RV--	-----V----
32	BoLA-DRB3**	H--EY	STS-------	-----F-D-Y	YT-G--T---	---W--F---	----PQ-R--	------F--S	K--E--RV--	----GM----

DQB sequences.

DQB-RFLP		10	20	30	40	50	60	70	80	89
		+| + + |	|	+ + |	++ |	|	+ |	+ + + + | + + |	
	HLA-DQB1*0501	YQ	FKGLCYFTNG	TERVRGVTRH	IYNREEYVRF	DSDVGVYRAV	TPQGRPVAEY	WNSQKEVLEG	ARASVDRVCR	HNYEVAYRG
1(A)	BoLA-DQB1*1A	--	----------	-----Y---Y	---Q--N---	---WDE----	--L---D---	F----DF--Q	T--EA-T---	---Q-EAPF
2	BoLA-DQB1*2	--	--C-------	-----Y---Y	---Q------	-----E----	--L---P--H	-----DF--Q	T--E--S---	---QLELIT
3A/C	BoLA-DQB1*3	F-	----------	-----L---N	---Q------	---W-E---L	--L---D---	-----DL--Q	T--EA-T---	---QAELIT
7A	BoLA-DQB1*7	--	----------	-----Y----	---Q--N---	---WDE----	--L---D---	-----DI--R	T--EA-T---	---Q-EAPF
9A/B	BoLA-DQB1*9	V-	----------	-----L-V--	-------A--	-----NE----	--L---D---	-----DL--Q	R--E------	---Q--APF
9A/B	BoLA-DQB2*1	V-	-M-Q------	-----Y---Y	---Q---A--	---W-E---L	--L---A---	-----DI--Q	TW-E------	N--Q-EAPF
12	BoLA-DQB1*12	F-	----------	-----S-N-Y	----------	---WDE---L	--L--QD---	-----DF--Q	T--EA-T---	---Q--APF
12	BoLA-DQB2*3	F-	-M-Q------	-----Y---Y	---Q--FA--	---WDE----	--L---D--L	-----DI--R	T--E------	N--Q-DAPF
1(B)	BoLA-DQB1*1B	V-	----------	-----S-N-Y	---Q------	---WDE---L	--L---D---	-----DI--R	T--EA-T---	*N--Q-DAPF*
4	BoLA-DQB1*4A	V-	----------	-----Y---Y	---Q--N---	---WDE----	--L---D---	-----DI--R	V--E------	N--QAELIT
4	BoLA-DQB1*4B	--	--C-------	-----Y---Y	---Q--N---	---WDE----	--L---D---	-----DI--R	V--E------	N--QAELIT
5	BoLA-DQB1*5	--	Y---------	-Q---L---Y	-------FA--	-----NEF---	--L---D---	-----DI--Q	R--E------	---QLDAPF
5	BoLA-DQB3*1	--	-IYQ------	-----S-KKQ	----Q-H---	----NEF---	S-L-QRD---	F--H/DF-KQ	T--E--T---	---QLELIT
6A	BoLA-DQB1*3	F-	----------	-----L---N	---Q------	---W-E---L	--L---D---	-----DL--Q	T--EA-T---	---QAELIT
7A	BoLA-DQB1*7	--	----------	-----Y----	---Q--N---	---WDE----	--L---D---	-----DI--R	T--EA-T---	---Q-EAPF
9A.1	BoLA-DQB-Q1	V-	----------	-----L-V--	-------A--	-----EE----	.....H---	-----DL--Q	R--E------	---Q--APF
10	BoLA-DQB1*9A	V-	----------	-----L-VG-	-------A--	-----NE----	--L---D---	-----DL--Q	R--E------	---Q--APF
10	BoLA-DQB2*2A	--	-M-Q------	-----Y---Y	---Q---A--	---WDE---L	--L---A---	-----DI--R	T--E------	N--Q-EAPF
	BoLA-DQB2*2B	--	-M-Q------	-----Y---Y	---Q---A--	---*WDE*---L	--L---A---	-----*DI*--R	T--E------	*N--Q-DAPF*
11A/C	BoLA-DQB1*11C	FR	---------R	-----Y---Y	T--Q--N---	---WDE----	--L---S---	F---EDL--R	T--E------	N--Q-EAPF
11A/C	BoLA-DQB2*4	--	-M-Q------	-----Y---Y	---Q--N---	---WDE----	--L---D---	F----DF--Q	T--EA-T---	---Q-EAPF
13C	BoLA-DQB13B									
	BoLA-DQB4*1	I-	--C-------	----QY---H	---Q--FM--	--EV-E-----	--L--RQT--	F----DE--R	V--E--T---	---QAELIT

Table 3. The mAb used in this study. For mAb 1 - 8, the bovine class II DRB3-PCR and DQB type positive for the key AA motive, as infered from the exon 2 sequences of DRB3 and DQB is given. For mAb 9 - 14, the bovine class II DRB3-PCR and DQB type positive for the mAb is given. The class II haplotypes present in the homozygous animals are in bold and underlined.

		postulated mAb bindingsite						
	HLA-mAb	Class II chain	AA number	AA residu	Ig-(sub) class	Ref	bovine DRB3-PCR	bovine DQB
M1.	NDS40	DRB1	37-38	YV	M	1	1, 2, 3, 13, **22**, 33, 35, 36, 37, 38	1.1B, **1.2**, **1.3**, **1.12**
M2.	JS1	DRB1	67-70	LLEQ	G_2	2	9	**1.3**, 1.9A, **1.9**, 1.Q1
M3.	24C7	DRB1,4,5	73	A	G_{2a}	3	all	all
M4.	TAL8.1	DRB1	9-13	EYSTS	G_2	4	**7**, 13, 16, 32, 39	none
M5.	TAL12.1	DQB1	52-53	PL	G_2	5	none	all
M6.	TAL14.1	DRB1	18	F	G_2	6	all	none
M7.	TAL15.1	DRB1,4	77	T	G_2	7	all but **11**, **23**, **24**, 27, 28, 32, 37, 38	**1.1A**, 1.1B, **1.3**, **1.7**, **1.12**, 2.4, 3.1
M8.	TAL16.1	DRB1,5	70	D	G_2	8	none	none
M9.	7.5.10.1	--			G_{2a}	9	**24**	??
M10.	7.3.19.1	DRB3,1*03			G_{2a}	9	none	none
M11.	IL-A21	--			G_{2a}	10	all	none
M12.	TH14B	--			G_{2a}	11	all	none
M13.	TH22A5	--			G_{2a}	11	none	all
M14.	TH81A	--			G_{2b}	11	none	all

References:

1. Bunce *et al.* 1990, 2. Sachs et *al.* 1986, 3. Cayrol *et al.* 1992, 4. Bodmer *et al.* 1984, 5. Marsh et *al.* 1992, 6. Madox *et al.* 1989, 7. Madrigal *et al.* 1989, 8. Sadler et *al.* 1993, 9. Koning et *al.* 1986, 10. Teale (personal communication), 11. Davies et *al.* 1987.

cattle and man), and selected to recognize cross-reactive determinants on MHC class II DR and DQ products (Davis *et al.* 1987). The mAb IL-A21 (a gift from Dr. A.J. Teale) was derived from mice immunized with bovine lymphocytes. This mAb was used for the 1D-IEF typing of bovine DR products in the FIBW (Davies *et al.* 1994).

Immunofluorescence and flow cytometry (FACS) analysis

Bovine PBMC were incubated with nine anti-HLA mAb, and binding was analyzed by one-color flow micrometry according to previous described techniques (Joling *et al.* 1994). The fluorescence staining was performed with a goat anti-mouse FITC conjugated immunoglobulin with specificity for gamma and light chains (GAM/FITC, Caltag, San Francisco, CA, 1:50). Anti-HLA mAb, 7.3.19.1 was used as negative control, and IL-A21 was used as positive control. For the analysis, a FACSscan (Becton Dickinson, Mount View, CA) was used. In each experiment 5.000 cells were counted and the percentage of the positive cells was calculated.

Complement-dependent cytotoxicity assay

The two-color fluorescence (TCF) method previously described (Van Rood *et al.* 1976) with minor modifications (Nilsson *et al.* 1994) was used.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

For the SDS-PAGE, precipitates were run overnight in a 12.5% polyacrylamide gel at 20 mA and a maximum of 120 V. Gels were fluorographed as described for 1D-IEF. Precipitation was performed with 3μl ascites or concentrated supernatant.

Immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF)

Immunoprecipitation and 1D-IEF was performed as described by Bissumbhar *et al.* 1994. Precipitation was performed with 3μl ascites or concentrated supernatant.

RESULTS

We hypothesized that eight polymorphic anti-HLA mAb were reactive with bovine lymphocytes on the basis of sequence homology with the HLA products recognised by the mAb. To test this hypothesis, first the reactivity of eight anti-HLA monoclonal antibodies toward bovine lymphocytes was analyzed by flowcytometry (FACS) and microcytotoxicity testing (TCF). Cells from cows proven to be homozygous for class I

and class II were used in this analysis. A monomorphic anti-HLA mAb previously shown to react with cattle lymphocytes was included in this analysis.
The results of the FACS and TCF analysis are summarized in Tables 4a and 4b respectively.

Table 4a. FACS-analysis of nine anti HLA-mAb (M1-M9), one negative control mAb (M10), and one positive control mAb (M11), using the cells of six homozygous animals.

ID	DH	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	@
1983	07A	---	---	+++	---	+++	+++	---	+++	---	--	++	
2399	08A	---	---	+++	---	---	---	+++	---	+++	--	++	
2337	11A	---	---	---	---	---	+++	+++	---	---	--	++	
2346	22B	---	---	+++	---	---	---	+++	---	---	--	++	
2202	23E	---	---	---	---	---	+++	+++	+++	+++	--	++	
2390	24A	---	---	---	---	---	+++	+++	+++	+++	--	++	

@ Used mAb: M1 NDS40, M2 JS1, M3 24C7, M4 TAL8.1, M5 TAL12.1, M6 TAL14.1., M7 TAL15.1, M8 TAL16.1, M9 7.5.10.1, M10 7.3.19.1, M11 IL-A21

Table 4b. TCF-analysis of nine anti HLA-mAb (M1-M9), one negative control mAb (M10) and one positive control mAb (M11). The reactivity of the mAb on the cells of seven homozygous animals is given as strenght of cytotoxicity.

		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	@
		---	---	---	---	---	---	---	---	---	--	--	
ID	DH	012	345	678	567	567	678	567	567	345	34	68	#
1983	07A	---	---	888	---	8--	888	---	---	---	--	88	
2399	08A	---	---	888	---	---	---	888	---	---	--	88	
2337	11A	---	---	4--	---	6--	886	888	---	---	--	88	
2346	22B	---	---	888	---	---	---	888	---	---	--	88	
2202	23E	---	---	666	---	886	888	888	886	888	4-	88	
2390	24A	---	---	---	---	---	886	84-	886	844	--	88	
2386	24A	---	---	---	---	6--	888	886	888	888	--	88	

@ See 4a.

Used dilutions: 0 (neat), 1 (1:10), 2(1:20), 3(1:10^2), 4(1:2.10^2), 5 (1:5.10^2), 6 (1:10^3), 7 (1:2.10^3), 8 (1:5.10^3)

Strenght of cytotoxicity: - = 0-20% cell killing; 4 = 20-40% cell killing; 6 = 40-80% cell killing; 8 = 80-100% cell killing

In both assays, the anti-HLA mAb, mAb1 (NDS40), mAb2 (JS1) and mAb4 (TAL8.1) were negative, whereas the other six anti-HLA mAb, mAb3 (24C7), mAb5 (TAL12.1), mAb6 (TAL14.1), mAb7 (TAL15.1), mAb8 (TAL16.1) and mAb9 (7.5.10.1) were positive. The reactivity in the FACS of the six positive anti-HLA mAb and IL-A21 is shown in Figure 1. The used dilutions of the anti-HLA mAb varied from 1:500 to 1:10.000. Comparison of the data of Table 4a and 4b show that (in general) the reactivity pattern of the anti-HLA mAb obtained in FACS and TCF towards the six homozygous animals were quite similar, except for mAb5 (TAL12.1).

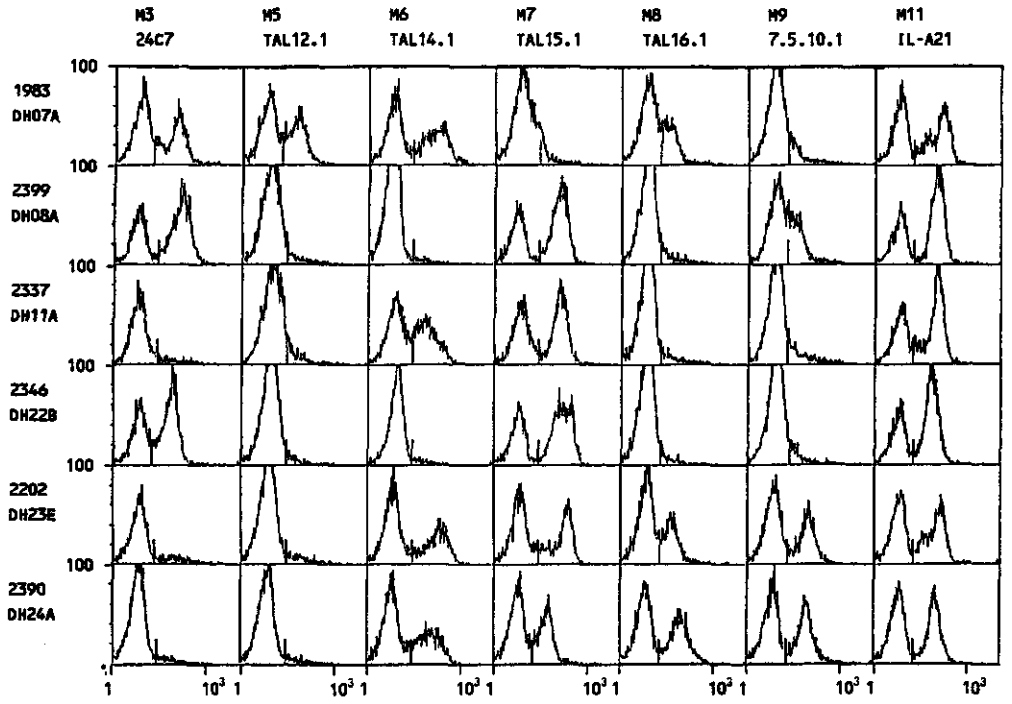

Figure 1. Analysis by flow cytometry of the binding of mAb 24C7, TAL12.1, TAL14.1, TAL15.1, TAL16.1 and 7.5.10.1, with lymphocytes of six cows proven to be homozygous for class I and class II. The monomorphic anti-BoLA class II mAb IL-A21 was used as positive control. The anti-HLA DR mAb 7.3.19.1 was used as negative mAb control, and FITC-rabbit anti-mouse Ig as a negative control. The histograms plot cell number against fluorescence intensity on a logarithmic scale; 5.000 cells were analyzed for each plot.

For this mAb the FACS and cytotoxicity data are not in agreement. The data of Table 4a and 4b not only show anti-bovine reactivity of the anti-HLA mAb, but also reveal that the reactions are polymorphic.

In addition to the six homozygous animals 55 heterozygous animals were typed in the TCF, and the cytotoxic reactivity of the mAb was deduced to reactivity per class II haplotype (DH). For some of the mAb - DH combinations, it was not possible to deduce with certainty the reaction as positive (+) or negative (-), and the reactivity was considered as doubtful (o) (Table 5). The outcome confirmed the data obtained with the homozygous animals. Moreover, it revealed information concerning the specificity of the anti-HLA mAb towards other class II haplotypes (Table 5).

Table 5. Overview of TCF-analysis of the HLA-mAb M3, M5, M6, M7, M8 and M9, based on the reactivity of the six homozygous animals and 55 heterozygous animals. The reactivity of the mAb is given as strenght of cytotoxicity in percentage of cell killing.

DH	N	M3	M5	M6	M7	M8	M9	@
07A	9	++	--	++	--	--	--	
08A	15	++	--	--	++	--	--	
11A	9	--	--	++	++	--	--	
22B	3	++	--	--	++	--	--	
23E	9	++	++	++	++	++	++	
24A	15	--	--	++	++	++	++	
03A	6	oo	++	--	oo	--	--	
11C	2	++	--	--	oo	--	--	
12C	6	++	++	--	oo	--	--	
16A	11	++	oo	--	++	--	--	
18A	9	++	--	--	oo	--	--	
22I	3	++	--	--	++	--	--	
22H	2	++	--	--	++	--	--	
26A	4	++	--	oo	oo	--	--	
27A	12	--	--	--	--	--	--	
28A	4	--	oo	++	++	++	--	
28B	1	--	oo	++	++	++	--	
32A	2	++	++	++	oo	++	++	

N Number of animals involved in the analysis
@ see table 4a
++ scores 6-8
-- scores 1-2
oo doubtful/not interpretable

All the TCF results, combined with the FACS data obtained with the homozygous animals, clearly demonstrated that in the various BoLA class II haplotypes the reaction patterns obtained did not correspond with those expected on the basis of the presence of the key AA responsible for epitope recognition (Table 6).

Table 6. Analysis of six anti HLA-mAb (M3 - M9) on the cells of six homozygous animals by 1D-IEF. The expected mAb reactivity (E) is given in the first column, and the mAb reactivity in FACS (F) and TCF (T) are given in the second and third column. The results obtained by immunoprecipitation and subsequent SDS-PAGE and IEF analysis (I) are given as reactivity for DRB3 (R) and DQB (Q).

	M3 (24C7)		M5 (TAL12.1)		M6 (TAL14.1)		M7 (TAL15.1)		M8 (TAL16.1)		M9 (7.5.10.1)	
DH	EFT	I RQ	EFT	I RQ	EFT	I RQ	EFT	I RQ	EFT	I RQ	EFT	I RQ
07A	+++	+−	++−	−−	+++	++	+−−	oo	−+−	−−	−−−	−−
08A	+++	+−	+−−	−−	+−−	−−	+++	+−	−−−	−−	−+−	−−
11A	+−−	−−	+−−	−−	+++	++	+++	oo	−−−	−−	−−−	−−
22B	+++	oo	+−−	oo	+−−	oo	+++	oo	−−−	oo	−−−	oo
23E	+−+	oo	+−+	oo	+++	++	+++	oo	−++	oo	−++	oo
24A	+−−	−−	+−−	−−	+++	+−	+++	+−	−++	+−	+++	++

(+) positive, (−) negative, (o) not done

The six anti-HLA mAb positive in FACS and TCF, were subsequently analyzed biochemically (SDS-PAGE and 1D-IEF), for their reactivity with DR and/or DQ products. The anti-BoLA mAb TH14B (DR), TH22A5 (DQ like) and TH81A ('DQ' like), used in previous biochemical analyses (Bissumbhar *et al.* 1994), were used as positive controls. In Table 6 the results of the biochemical analysis are summarised. TAL12.1 was negative in both SDS-PAGE and 1D-IEF. The remaining five mAb all recognized DR-associated products as revealed by the similarity in banding pattern obtained by 1D-IEF analysis using both TH14B and the mAb in question. Notably, precipitation with TAL14.1 also resulted in a reaction pattern similar to that of TH22A5 (DQ-like), including the non-precipitation of products from DH24A haplotype. Precipitations with 7.5.10.1, revealed a pattern resembling that of TH81A in addition to DR precipitation. Interestingly, all DR precipitating mAb only had

affinity for the 'upperband' of the previously DRB associated 'doublet' as obtained by TH14B or by IL-A21 precipitations.

The presence of the sequences similar to the HLA key AA motif within exon 2 of bovine DRB3 and DQB, appeared to be not related with the reactivity of the anti-HLA mAb. For three mAb however, alternative AA motifs that may influence epitope recognition were detected. Absence of Arginine at position 77 on DRB3 exon 2 seemed to be positively associated with binding of 24C7, and negatively with binding of TAL14.1. Comparison of the reactivity pattern of these two mAb with the serological and 1D-IEF DRB3 defined specificities, revealed that TAL14.1 was notably negative for the Ds01 positive animals, which have the biochemical specificities DRBF4, DRBF5, DRBF6, and DRBF7, whereas mAb 24C7 seemed to be negative for Ds03 and Ds13 positive animals. Positive reactivity of TAL16.1 (70:D) in cattle, was associated with DRB and the presence of Glutamic acid at position 70 on DRB3 exon 2.

DISCUSSION

The objective of this study was to investigate, if the sequence homology in epitope regions between man and cattle, as predicted from the available bovine DRB and DQB exon 2 sequences, can be used in the search for mAb specific for bovine class II molecules. The analysis by FACS and TCF revealed anti-bovine reactivity of six anti-HLA mAb. Discrepancy between the results obtained with the two assays was observed. This might be due to differences between the assays. In the TCF, lysis occurs due to complement binding to the Fc receptor of the Ig molecules, whereas in the FACS analysis, also aspecific binding may give a positive signal.

In the biochemical analysis, no precipitation of bovine molecules was detected for TAL12.1, although this mAb did show positive reaction in the FACS and TCF. It may also be that TAL12.1 recognizes a determinant present on the class II chain, which is conformationally altered during the biochemical analysis. The five other anti-HLA mAb precipitate bovine MHC molecules. Based on the similarity of 24C7, TAL14.1, TAL15.1, TAL16.1 and 7.5.10.1 with the focusing pattern of the TH14B precipitated products, we suggest that these anti-HLA mAb precipitated DR molecules. Precipitation of just the upper band of the previously defined DRB associated doublet by the anti-HLA mAb may emphasize the specificity of the mAb.

TAL14.1 revealed not only banding patterns similar to TH14B, but also a pattern highly comparable with that obtained by TH22A5. Even absence in the 1D-IEF of DQA products for the haplotypes DH07A, DH11A, and DH24A (haplotypes with a single DQA W1 gene) as described for TH22A5 (Bissumbhar *et al.* 1994) was observed for the TAL14.1 precipitation. Based on these data we conclude that TAL14.1 precipitates both DR and DQ molecules, whereas the DQA products precipitated by TAL14.1 are likely the products of the DQA A5 locus.

The banding pattern obtained with 7.5.10.1 was comparable with that obtained with both TH14A and TH81A, which might indicate an anti DQ reactivity of 7.5.10.1. in addition to DR. However, precipitation with 7.5.10.1 was only performed with DH24A positive cells. Precipitation with DH23E or DH32A positive cells has to be performed to confirm the TH81A associated anti DQ reactivity of 7.5.10.1.

The reactivity of the mAb used in this study differed completely from the expected reactivity (Table 6). Screening of the DRB3 and DQB exon 2 sequences for the occurrence of putative key AA sequences, confirmed that the key AA responsible for epitope recognition were not associated with the actual observed reactivity of the mAb. It should be noted that although the procedure by which we have linked the known DRB and DQ sequences to the haplotypes present in our animals ensures a high degree of reliability, we cannot fully exclude the possibility that mutational events have disrupted, or conversely, have created putative AA motifs for epitope recognition. In fact, the only way to be absolutely sure is to use sequence based typing techniques, such as oligonucleotide-typing (PCR-SSO), but unfortunately these have not been developed for the bovine MHC.

Comparing the expected and observed reaction pattern of the anti-HLA mAb, we were not able to define AA which might be responsible for binding of TAL12.1 and TAL15.1. For 24C7, Alanine at position 73 on the DRB chain was postulated to be responsible for epitope recognition of the mAb. As both HLA-DRB and HLA-DQB are positive for 73:A, restriction to DR is likely determined by a DR specific epitope, which is conserved in man difference between DR and DQ molecules in tertiary structure, likely cause by differences in conserved AA, for example at position 51 (DRB:E, DQB:P). We suggest that also in cattle reactivity of 24C7 is restricted to DRB, likely due to structural differences between BoLA-DR and -DQ, which might be similar to those in man. In cattle however, the reactivity of 24C7, is negatively influenced by the presence of Arginine at position 77. In man, Arginine does not occur at position 77.

The definition of the key AA of TAL14.1 was based on the non-responsiveness of TAL14.1 with HLA-DRB4. As HLA-DRB4 differ at position 18, 41, 44, 48, or 81 from the other HLA-DRB alleles, at first instance one would suppose TAL14.1 to be restricted by these five AA. But, since TAL14.1 reactivity is restricted to HLA-DRB, reactivity of TAL14.1 was suggested to be restricted by the presence of Phenylalanine at position 18 of HLA-DRB molecules because the AA at position 41, 44, 48 and 81 are identical for HLA-DRB and DQB.

In cattle, TAL14.1 displayed reaction with both DR, and with DQ molecules. Obvious, the differences between HLA-DRB and HLA-DQB restricting the anti-HLA reactivity of TAL14.1, is not present in cattle. As both HLA-DQB and BoLA-DQB are positive for Threonine at position 18, restriction to HLA-DRB is not determined by this AA residue. Neither is the anti-bovine reaction pattern determined by the AA at position 41, 44, 48, or 81. Furthermore, no AA alteration associated with the absence of DQ specific bands in the 1D-IEF for DH24A could be detected. We suggest that the AA at position 18, 41, 44, 48, or 81 are not the only AA residues important for binding of TAL14.1. Additional AA are important for the epitope recognition of TAL14.1, but we were not able to determine which.

For TAL16.1 the postulated key AA site in man is Aspartic acid (D) at position 70. In cattle Aspartic acid never occurs on position 70. Still anti-BoLA reactivity of TAL16.1 was observed, which was restricted to cells positive for Glutamic acid (E) at position 70. Glutamic acid and Aspartic acid both have an acidic side chain, which only differ for a single carboxyl group. Replacement of 70:D for 70:E , therefore is a conserved substitution, which does not occur in man. So, we suggest that in cattle 70:E is the key AA determining the binding of TAL16.1.

The reactivity pattern of 7.5.10.1 was almost identical to that of TAL 16.1. However, this mAb revealed monomorphic reactivity in man (DRB, DQB and DPB) and dog (DRB). Therefore, it is likely that this mAb reacts with a conserved region on the MHC molecules. If the AA motif for binding of 7.5.10.1 is located in exon 2 of class II B-chains, it might be that the AA important for recognition is/are located between AA position: 40-52, as this region is conserved between man DRB, DQB, DPB, canine DRB and bovine DRB3 exon 2. According to this hypothesis, the change of 46:G in 46:D,N or E, and 47:F in 47:Y prevents binding to bovine DQB (see Table 2). Restriction to DRB3 of DH24A might be due to presence of 30-32:YYT - 37:T, instead of 30-32:CFH - 37:F, although variance at position 70 and 74 may be involved as well.

Based on the anti class II reactivity observed in this study, we suggest that the discrepancy between observed and expected reaction patterns might be caused by the large evolutionary distance between man and cattle, i.e. the mAb binding might be disturbed due to some evolutionary conformational changes of parts of the class II molecules. Compared to chimpanzee, where the mAb behaved as expected (Marsh *et al.* 1992), cattle is much less closely related to man, the evolutionary distance between cattle and man is 75 million years, and between chimpanzee and man is only 7 million years.

It may also be that the bovine (self-)peptides binding in the groove have negative influence on the binding of the anti-HLA mAb. If this is true, binding with bovine class II molecules by mAb which have an AA motive in the ß pleated sheet (NDS40, TAL8.1, TAL14.1), might be hampered by the presence of bovine (self-)peptides, or variances in the AA-lining in the groove, as the key AA sequence is of these mAb is located in the bottom of the groove. In accordance with this hypothesis, mAb NDS40 and TAL8.1 were indeed negative. TAL14.1, however, displayed an excellent response. If Phenylalanine at position 18 is important for epitope recognition by TAL14.1, in this particular case, it might be that TAL14.1 is able to react, because the AA motif of TAL14.1 (18:F) is located on a (non-polymorphic, conserved) protruding part of the ß pleated sheet. In contrast, the five anti-HLA mAb that have a AA motif in the α helical stretch, all reacted with the bovine lymphocytes, although, under the conditions tested, mAb JS1 reacted only weakly.

In this study cross-reaction of anti-HLA mAb with bovine class II molecules has been studied, and animals, homozygous for BoLA class II have shown to be very instrumental in this. The results clearly demonstrated that anti-HLA mAb can detect bovine class II polymorphism. However, the data revealed that sequence homology in the epitope region is not always sufficient for cross-species recognition by anti-HLA mAb. Although, sequence data are lacking to confirm the observed anti-BoLA reactivity, and define the AA responsible for recognition of the BoLA-epitopes, this study already shows that the use of non-human class II MHC positive cells can provide additional information on anti-HLA mAb reaction patterns.

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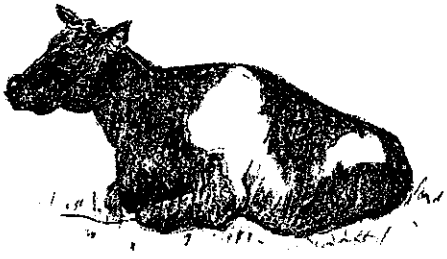

Chapter 8

GENERAL DISCUSSION AND CONCLUSIONS

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In this thesis an analysis of bovine MHC class II haplotypes is described, with emphasis on the development of class II serology. At the time this study started, only limited knowledge on the BoLA class II region was available, and the serotyping for class II was still in its infancy. For reasons of clarity, first the development and improvements of BoLA class II serology will be discussed and subsequently, the more general aspects of bovine class II MHC typing will be reviewed.

1. BoLA class II serology

Serology reflects the reactivity of the humoral immune response towards antigenic determinants on MHC molecules. Based on this reactivity, expressed MHC polymorphism can be defined. As in HLA, we assumed that bovine class II serology primarily reflects the recognition of determinants present on the MHC molecules encoded by DR and DQ genes. The detection of, and distinction between the products of these two class II loci is important, since both are involved in regulation of the immune response. Moreover, broadening of our insight in the expressed bovine class II polymorphism, will also contribute to the overall understanding of the bovine MHC.

Serum production

In cattle, class I serotyping already had been available for several years, and production of class I sera had been shown to be relatively easy (Bernoco *et al.* 1991, Davies *et al.* 1994). Therefore, the immunisation method used for production of class I sera was also applied for production of class II sera. A total of 49 immunisations for serum production were performed, which resulted in 28 class I sera, and (only) 18 class II sera (Chapter 2). This shows a limited success in the production of anti-class II sera. The cause of this is not known. One can only speculate about it. Class I molecules are present on each cell, whereas class II molecules are only present on a subpopulation, which may result in presentation of a lower number class II molecules in the immunisations by skin transplantation. It may also be, that the nature of the barrier across which antibody production is elicited, influences the success rate. For instance, production of a class I anti A10 serum fails

across an A11 barrier, but is successful across an A14 barrier, and production of a class II anti Ds04 (DH18A and DH26A) serum fails across the DH07A and DH11A barrier, but is successful across the DH24A barrier. However, this may not be the case for each class II barrier, as a Ds11 serum was produced between a donor - recipient pair, both positive for Ds03 (Chapter 4). We suggest that poor information on the donor and receptor animals, has hampered the production of class II sera, as a small rise in 'success rate' (Chapter 2, in the first group 5 out of 18 immunizations were positive, in the second group 6 out of 16, and in the third group 7 out of 15) coincided with the increasing information on the MHC haplotypes of the animals.

Based on the outcome of the screening of the class II alloantisera (Chapter 3), two additional aspects are worth noting. First, since the class II sera produced in Wageningen clustered very well with the 41 sera donated by Davies, Lazary and Spooner, the choice of method used to produce class II alloantisera (class II compatibility and no absorption, versus class II incompatibility and platelet absorption), was shown to be of minor importance. Second, the screening revealed that in some cases sera produced in different breeds (Simmentaler, Angus, Holstein Friesian), can have a similar specificity. We suggest that these specificities are associated with the more ancient/conserved epitopes of the class II molecules.

Discrimination of anti-DR and -DQ antibody populations

At the time immunisations were performed to produce class II alloantisera, little was known about the class II haplotypes carried by the donor and the receptor animals (Chapter 2, Table 1). Due to deficient class II definition, polyclonal responses against multiple determinants on distinct molecules encoded in the class IIa region were induced. To detect to what extent the bovine anti-B cell alloantisera were mixtures of different antibody populations, and to what extent this influenced the definition of the class II polymorphism, sera were analyzed in absorption studies and by MAILA (Chapter 4). It appeared that the sera were indeed mixtures of different antibody populations and evidence for the presence of distinct DR or DQ specific antibody populations was obtained.

Discrimination between the bovine DR and DQ specific antibody populations was complicated for two reasons. First, there is a strong linkage disequilibrium between DR and DQ (Sigurdardóttir *et al.* 1988), and second, as mentioned before, the limited knowledge of the genotypes of the animals at the time the absorption was performed, caused mis-interpretations, as illustrated by the following example.

A dam-offspring combination (1981-2378) used in the study described in Chapter 5, was Ds15 and DRBF08 positive, and therefore assigned as DH28A positive. Both animals were also positive for Ds11, but at that time the Ds11 reactivity was considered to be false positive. After typing for DQ by 1D-IEF (Chapter 5) and by RFLP (Chapter 6) however, the haplotype definition for these two animals (and their relatives) appeared to be wrongly assigned, and they were re-defined as DH28B positive (Chapter 6, Table 1). Because of the simultaneous reactivity of Ds11 and Ds15 defining sera, animal 1981 was used for absorption of a Ds11 positive serum (W2245). In Table 1 the results of this absorption are summarised. In addition to what is described in Chapter 4, in Table 1 the typing results of 1D-IEF for DRB3 and RFLP for DQ were added to the serological class II typing data. Without this extra information the outcome of the absorption performed with animal 1981 is very difficult to interpret. The addition of this extra information, showed that the specificity Ds11 contains two antibody populations, one associated with the 1D-IEF type DRBF07 (DRB3-specific), and a second associated with RFLP type DQA*1B-DQB*1 (DQ-specific).

Table 1. Reaction pattern detected in the TCF of serum W2245 (anti Ds11) before (O) and after lymphocyte absorption (A, B, C, D, E, and X). Lymphocytes used for absorption were isolated from the animals 0513 (A), 2390 (B), 2323 (C), 2297 (D), 2346 (E) and 1981 (X). The reaction with B-lymphocytes of these six animals is given.

PANEL CELLS								SERUM W2245						
	haplotype-1			/	haplotype-2				lymp. absorbed					
CELL ID	SER Ds	DRB3 IEF DRBF	DQ RFLP A-B	/	SER Ds	DRB3 IEF DRBF	DQ RFLP A-B	O 67	A 56	B 56	C 56	D 56	E 56	X 56
A-0513	03-11	07	1B-1	/	02	02	2-2	88	--	88	88	88	88	88
B-2390	03-10	03	1A-1	/	03-10	03	1A-1	--	--	--	--	--	--	--
C-2323	03-15	08	7A-7A	/	01-14	06	9A-9A	--	--	--	--	--	--	--
D-2297	01-12	06	12-12	/	01-12	06	12-12	--	--	--	--	--	--	--
E-2346	01-09	07	9B-9B	/	01-09	07	9B-9B	--	--	--	--	--	--	--
X-1981	03-11	08	1B-1	/	03-15	08	7A-7A	88	--	88	6-	88	88	--

Strength of cytotoxicity:
- = 0 - 20% B-cell killing, 6 = 40 - 80% B-cell killing, 8 = 80 - 100 % B-cell killing.

The anti-DQ reactivity was not very consistent. Probably the titre of the DQ specific antibody population was much lower than that of the DR specific antibody population. However, the condition of the test cells may also influence the expression of DQ, and consequently the detection of DQ by the alloantisera.
A similar situation as described for specificity Ds11, may be the case for alloantisera of other specificities, e.g. the alloantisera used to define Ds08. Absorption studies, if possible, combined with the MAILA assay are needed to give definite answer in this matter.

In conclusion: the data obtained by serum absorption confirmed that the bovine alloantisera are complex mixtures of antibody populations, and it was conclusively shown that they contain antibody populations directed against DR and DQ specific determinants. When alloantisera are analysed for DR and DQ specific antibody populations, the availability of well typed animals, preferably with rare combinations of DR and DQ, is indispensable.

Analysis of serum specificities

The presence of both DR and DQ specific antibody populations complicates the interpretation of the reaction patterns of the BoLA anti B-cell alloantisera. A similar situation was also observed in man, where interpretation of reaction patterns of alloantisera containing both HLA-DR and DQ antibodies appeared to be difficult (Schreuder et *al.* 1984). One strategy to define the specificities of alloantisera is the use of other class II typing methods in the analysis. We started by comparing the serological definition of class II polymorphism with data obtained by 1D-IEF (Chapters 3 and 5).

Biochemical characterization of bovine DRB3 allelic variants was routinely performed. In addition, discrimination between expressed DQ allelic variants became possible, after modification of the standard 1D-IEF method used for DRB3 typing. The (albeit limited) definition of expressed DQ polymorphism by 1D-IEF analysis appeared to be very informative. Even with the restricted number of variants established, it became apparent that the 1D-IEF analysis of DQ enabled more extensive discrimination between the haplotypes defined by the combination of class II serology and 1D-IEF DRB3 typing. Unfortunately, the two mAb assumed to be bovine DQ specific, displayed cross-reaction with molecules encoded by DR, and therefore discrimination between expressed DR and DQ polymorphism remained complex (Chapter 5).

Half-way through the project several DNA based methods became available for BoLA class II typing. The DRB3-PCR RFLP, and the DQA and DQB RFLP typing was performed and included in the analysis (Chapter 6). Subsequently, on the basis of the results of the DNA typing, for a number class II haplotypes the amino acid sequences for DRB3 and DQB exon 2 were deduced from the published sequences (Chapter 7). Also, the DQA gene-types (W1 or A5) (Sigurdardóttir *et al.* 1991), the DQB gene-types (DQB1 - DQB4), and the DQB 3' primer types (LA40/LA41 or LA40/LA48) (Sigurdardóttir *et al.* 1992) were deduced. The additional data associated with the defined DQ polymorphism are shown in Table 2.

Table 2. Class II haplotypes (DH) sorted by class II serotypes, combined with the DQA-DQB typing data. The last three columns present additional information on DQ polymorphism, derived from Sigurdardóttir *et al.* 1992.

CLASS II HAPLOTYPE						
DH#	Class II serology (Ds)	DQA RFLP	DQB RFLP	DQA genes	DQB primer @	DQB genes @@
08A	01-12	12	12	W1+A5	P1+P1	1*12, 2*3
03C	01-17-14-06	12	12	W1+A5	P1+P1	1*12, 2*3
03A	01-17-14-06	10	10	W1+A5	P1+P1	1*9, 2*2A
32A	01-17-14-05	9A	9A	W1+A5	P1+P1	1*9, 2*1
11C	01-17-14	9A	9A	W1+A5	P1+P1	1*9, 2*1
10C,16A	01-17-08	11B,A	11A,C	W1+A5	P1+P1	?1*11A,C, 2*4?
22I	01-09	11A	11A	W1+A5	P1+P1	?1*11A, 2*4?
22B,H	01-09	9B	9B,A	W1+A5	P1+P1	?1*9, 2*1
12C	01-16-05	13B	13C	A5+A5	Px	?x*x
18A	01-16-04-07	5	5	A5+A5	P1+P2	1*5, 3*1
26A	16-04	6A	6A	A5+A5	P2+Px	1*3, x*x
22K	Blanco	3C	3C	W1	P2	1*3
22F	09	3A	4	W1	P2	1*4A/B
07A	02	2	2	W1	P2	1*2
11A	13	3A	3A	W1	P2	1*3
24A	03-10	1A	1	W1	P1	1*1A
27A	03-11	1B	1	W1	P1	1*1B
28A,D,E	03-15	7A,D,G	7A	W1+A5	P1+Px	1*7, x*x

\# Haplotypes 12D, 22I, 23B, 23C, 28B are not taken into account, because for these haplotypes the definition was based on 'sire-haplotypes', detected in only one or two animals (see chapter 6).

@ Type of primer DQB genes P1 = LA40/LA41 and P2 = LA40/LA48 (Sigurdardóttir *et al.* 1992), Px = unknown

@@ x*x : DQB gene-type unknown

When all data on class II polymorphism obtained in the different techniques were compared, the high concordance between serologically defined class II haplotypes and the class II definition obtained by 1D-IEF DRB3 and DRB3-PCR RFLP typing (Chapter 3, Chapter 6) was most striking. A division of the serologically defined haplotypes into three main groups: 'Ds01 positive', 'Ds03 positive', and 'Ds01-Ds03 negative' was less clearly seen. This division was based on the following associations. First, as summarised in Table 2, all 'Ds01 positive' haplotypes, except for DH18A, were positive for the DQB gene DQB2, and share the same DQB 3'primer type (LA41) and primer combination (P1+P1). DH18A was positive for DQB gene DQB3. From the AA sequences of DQB2 and DQB3, it appeared that only these two DQB gene-types had glutamine at position 14 (14:Q) in common. Second, the 'Ds01-Ds03 negative' haplotypes were positive for the same DQB 3'primer type (LA48). In agreement herewith, all the 'Ds01-Ds03 negative' haplotypes were 84-89:QAELIT positive. Third, both the 'Ds03 positive' haplotypes shared unique fragments in the RFLP analysis for DQA and DQB polymorphism.

These three main groups exhibit a striking similarity to the first observations in man using B-cell alloantisera, where three specificities of B-cell alloantisera were defined as MB1, MB2 and MB3 (Duquesnoy *et al.* 1979). In man, it has now been accepted that the immunogenic composition of the class II haplotypes, where several DR specificities share the same DQ specificity, causes the formation of three groups. In conclusion, we hypothesize that the division of the serologically defined bovine class II haplotypes into three main groups is associated with DQ polymorphism. Some of the subspecificities observed in cattle, may be determined by DR polymorphism. This hypothesis is supported by the results obtained in the absorption study described in Chapter 3.

Class II serology and DRB3-PCR RFLP

Some alleles of DRB3, are difficult to distinguish by DRB3-PCR RFLP typing, whereas serological typing reveals a clear difference at the product level of these alleles. Two examples are summarised in Table 3. First, in the case of the types DRB3.2*07 and DRB3.2*08, the banding pattern on which the definition of these two types is based, is almost identical. The distinction is only based on the presence of one deletion at position 65 for the DRB3.2*07. Second, in the case of types DRB3.2*22 and DRB3.2*23, the distinction is only based on the presence of one extra restriction site for *Rsa I*. These two examples illustrate that typing errors can be easily made with DRB3-PCR RFLP, due to interpretation mistakes, and/or lack of

Table 3. Comparison of serological specificities with DRB3-PCR RFLP types.

Class II serology (Ds)	PCR-RFLP DRB3.2 allele	PCR-RFLP patterns *Rsa I*	*BstY I*	*Hae III*	
Ds02	07	e	c	c	@
Ds01-12	08	f	a	a	
Ds01-09	22	m	b	a	@@
Ds03-15	23	n	b	a	

@ Difference between *Rsa I* pattern 'e' and 'f', and between BstY I and Hae III pattern 'c' and 'a' is a deletion of three nucleotides at position 193-195, resulting in a fragment difference of three nucleotides.

@@ Difference between *Rsa I* pattern 'm' and 'n' corresponds with the presence of one extra restriction site in pattern 'm'.

resolution. Furthermore, the second example illustrates, that there are cases that one nucleotide alteration is associated with quite a difference in MHC polymorphism as detected by serology and/or 1D-IEF. Such cases may be indicative for the location of epitopes that are important in the immune response.

Some alleles of DRB3 can not be distinguished at all by DRB3-PCR RFLP typing, whereas other class II typing methods such as serological or biochemical typing can, e.g. DH11A and DH11C. Furthermore, recently, a study performed in Japanese breeds, revealed a DRB3-allele that was undetectable with DRB3-PCR RFLP method (Morita *et al.* 1994). Therefore, detection of DRB3 polymorphism should preferably not be based on the DRB3-PCR RFLP method alone. A second method for typing should be used, e.g. the DRB3 microsatellite PCR (Ellegren *et al.* 1993) technique.

2. General aspects

Developments

At the time this project started, many studies were underway on the influence of the MHC on various diseases and a very optimistic view of the importance of MHC in genetic control of immune response stimulated many researchers to study the MHC in livestock. It was suggested that the MHC could be used as a tool in breeding for disease resistance, and numerous MHC-disease association studies in livestock were

started. In cattle however, performance of disease association studies in which MHC class II was involved was hampered by limited knowledge on BoLA class II polymorphism.

Now five years later, enormous progress has been achieved in the definition of bovine class II. There is now a good basis for the definition and unravelling of the genetics of MHC, and this will facilitate MHC-disease association studies in cattle. Moreover, two DNA based methods are available for routine typing of DRB3 polymorphism (DRB3-PCR RFLP and microsatellite typing) of large numbers of animals.

At the same time that knowledge of the bovine class II MHC typing was accomplished, also insight in its relation to disease has been changed considerably. From several livestock studies, e.g. Marek's disease in chickens (Pinard *et al.* 1991), it became clear, that not only MHC (or MHC-linked) genes, but also non-MHC effects, are important in the genetic control of disease resistance. In accordance with these new data, the ideas about the immediate practical application of MHC - disease association studies in livestock species have become more realistic. In addition to this, the expectations are that within a few years genetic maps will be available for cattle. These maps will form the basis for identification and location of non-MHC loci associated with disease resistance or production traits.

In association studies, identification of class I and/or class II haplotypes by DNA based typing methods will be sufficient. However, for studies concerning immune responses and antigen presentation, it remains important to know how and where MHC products are expressed. A correlation between DRB3 exon 2 AA sequence 70-71:ER and resistance to Persistent Lymphocytosis caused by Bovine Leukemia Virus has been described (Xu *et al.* 1993). It is unclear what functional mechanism lies at the basis of this association. The anti-HLA mab analysis (Chapter 7), may add some additional information. It appeared that mAb TAL16.1 reacted specifically with the haplotypes positive for glutamic acid at position 70 on DRB3 exon 2 (70:E), which indicates that the DRB3 polymorphism present at DNA level is also detected at the product level. As 70:E is positioned at an antigen recognition site, according to the proposed model of the class II molecule (Brown *et al.* 1993), it may be important in antigen presentation. Evidence for a role of glutamic acid in the immune response may be provided in a future study on T cell responses to BLV peptides. In this particular case, the antibody specificity seems to be more directly informative than the polymorphism detected by 1D-IEF, which illustrates the usefulness of serological detection of MHC polymorphism. The link with MHC polymorphism expressed at the

cell surface should be made when describing associations of DNA based MHC polymorphism with disease resistance/susceptibility or other immune response phenomena.

Application of BoLA typing

In developing class II serology and improving BoLA class II MHC definition, more than 600 animals were MHC typed (Chapter 6). This enabled the evaluation of the degree of MHC polymorphism as found in the WAU population. Moreover, since the typing was performed over a 5 year period, we were able to compare distribution of class I - class II MHC polymorphism over consecutive generations. The frequency distribution presented in Chapter 6 revealed an interesting aspect concerning the class II haplotype DH24A. It appeared that DH24A was present at relative high frequency in the WAU herd over three generations. Since this class II haplotype has been described to be associated with susceptibility for clinical mastitis (Lundén *et al.* 1990), the question was raised why this class II haplotype was present at a consistent high frequency. One would expect that, due to increased risk, female DH24A positive animals should have been selected and culled from the herd, resulting in a relatively low frequency of DH24A. But evidently this was not the case.

In Chapter 6, two MHC haplotypes positive for DH24A (A11-DRB3*24 and A19-DRB2*24) were suggested to be conserved haplotypes. In man, occurrence of combinations of MHC alleles in one haplotype at frequency significantly higher than expected from random association, is defined in several ways. Awdeh *et al.* (1983) defined this phenomenon as 'extended' haplotype. It appeared that one of their extended haplotypes displayed a segregation distortion. This MHC haplotype was transmitted from males to 83% of their offspring (Awdeh *et al.* 1983). Segregation distortion involving MHC genes has also been described in pigs (Philipsen and Kristensen 1985) and horses (Baily 1986), although without information on the type of MHC haplotype. Analysis of the BoLA class II typing data obtained in the WAU herd, revealed that segregation distortion may occur in Holstein Friesians as well. Based on 237 segregations (17 sires), DRB3-PCR RFLP type DRB3.2*24 (DH24A) was found to be transmitted from sires to 60% of their offspring, which is a frequency that is significantly higher than expected. Only one other DRB3-PCR RFLP type had a significant transmission bias from sire to offspring: DRB3.2*28, but this was only based on 80 segregations (6 sires). The other 15 DRB3-PCR RFLP types tested had no transmission bias. Although further statistical analysis should be performed, we now

suggest that in case of DH24A the transmission bias might be involved in maintenance of the consistent high frequency of DH24A in the WAU animals.

This example indicates that in future studies concerning MHC polymorphism in cattle special notice should be taken of the occurrence of these conserved haplotypes. The occurrence of the fixed combinations of MHC alleles, has to be taken into account because the polymorphism of the total, extended MHC region may be more important than that of the individual alleles.

Class I and class II homozygosity

When all results obtained by the different techniques were compared, 18 animals were defined to be homozygous for class I and class II (Chapter 6). However, only after typing of related animals, the animals were truly proven to be homozygous for their MHC. This shows clearly the value of the availability of a MHC typed herd with well defined pedigree structure.

In functional studies on bovine immune response absolutely the availability of well typed homozygous cell lines/PBMC is required. Indeed, the animals homozygous for their MHC appeared to be very instrumental in the study on reactivity of the anti-HLA mAb in cattle (Chapter 7). Also in a recent study on bovine T cell responses towards synthetic peptides, designed to be used as vaccine components homozygous animals were very helpful (Van Lierop *et al.* 1994). In addition hereto, homozygous animals are also very valuable in studies on the influence of MHC on reproduction. Such studies are underway.

The availability of MHC typed animals allows the design of specific breeding protocols to quickly produce MHC homozygous offspring, that can be used in many studies for MHC function. This approach is used by Gassbare (1994), who has produced a herd of Angus cattle homozygous for class I and class II, and subsequently selected the animals with an identical MHC for a study concerning reduced resistance to gastrointestinal nematodes.

General Conclusions

In this study anti-class II sera we produced (Chapter 2) and class II specificities were defined (Chapter 3). Subsequent analysis of the sera by absorption studies (Chapter 4) and application of the MAILA (Chapter 4) revealed that the alloantisera were complex mixtures of DR and DQ specific antibodies. Furthermore, serological discrimination between DR and DQ was proven to be possible. Subsequent application of biochemical (Chapter 5) and DNA based techniques (Chapter 6)

revealed extended insight in the serologically defined class II specificities. To achieve analysis of the serum specificities, extensive typing of a large number of cows had to be performed, which resulted in additional information on the distribution of the defined haplotypes in the WAU herd (Chapter 6). Finally, the extended typing of the bovine class II, appeared to be useful in the analysis of anti-HLA mAb reactivity in cattle (Chapter 7).

With the data reported in this thesis, further insight in the complexity of the bovine MHC has been obtained. Although the alloantisera are mixtures, and the serum panel is limited, typing by serology for BoLA class II is operational now. Moreover, detection of expressed bovine MHC class II polymorphism by serology proved to be very informative. Furthermore, with the present knowledge on bovine MHC class II polymorphism and with the methods available to date, the use of animals with a well defined MHC typed pedigree structure has strong preference.

In case MHC class II polymorphism is used as a genetic marker, DNA based methods are advisable for typing, and serology is not the method of choice. A combination of two (or more) DNA based typing methods may be used, e.g. the combination of DRB3-PCR RFLP and microsatellite typing, or the use of a single method applied for the detection of two loci, e.g. the use of two closely linked microsatellites in the DRB region. This option has recently been described as a convenient method for routine typing of DR haplotypes (Gwakisa *et al.* 1994). Usage of a combination of two microsatellites may also be convenient in typing of the complete MHC haplotype: one microsatellite associated with class I, e.g. the microsatellite near CYP21 (Chung *et al.* 1986), combined with a microsatellite associated with class II, e.g. the microsatellite near DRB3 (Ellegren *et al.* 1993).

However, in studies concerning the immune response, in addition to all the DNA based typing, class II serology (together with 1D-IEF) should be used to detect expressed MHC class II polymorphism.

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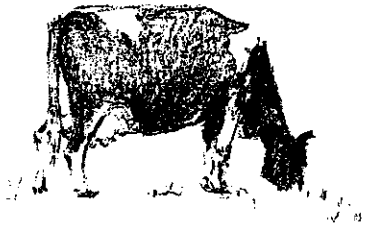


SUMMARY

In cattle there is an apparent variability in immune response towards infectious agents, in part, determined by individual differences in genetic resistance against infectious agents. Inclusion of genetic resistance against economically important diseases in breeding programs may contribute to a better control of infectious diseases. That application requires the identification of genetic markers for disease susceptibility and/or immune-mediated resistance, and the major histocompatibility complex (MHC) has been suggested to be an excellent candidate.

The MHC is a chromosomal region, containing a series of extremely polymorphic genes. The MHC genes encode for molecules which are present on the cell surface. These MHC molecules are important for the immune response, since they bind immunogenic peptides and present them to the immune system. Two classes of MHC molecules are distinguished: class I, present on all nucleated cells, and class II, only present on a small group of cells directly involved in the immune response. The MHC genes are expressed co-dominantly, meaning that the products of both homologous chromosomes (paternally as well as maternally inherited genes) of an individual are present on the cell surface. Each individual has its 'own' unique combination of MHC alleles, because the MHC genes are so polymorphic (numerous alleles per gene), except of course related individuals.

Numerous associations between disease susceptibility and MHC had already been described in man at the time this study started. Notably, due to the characterization of the class II genes, the significance of and the number of publications on MHC associated diseases have increased. Also, in cattle several MHC-disease associations have been reported. However, in those studies only class I polymorphisms were used as markers, because the knowledge on the genetic organisation and polymorphism of the bovine MHC class II region was very limited.

The above mentioned aspects were the major impetus for the study described in this thesis: an analysis of bovine class II polymorphism, which is essential for identifying the role of class II in disease susceptibility in cattle.

Several methods are available for detecting (typing) the MHC polymorphisms of an individual. MHC typing may be performed at the gene product level (MHC molecules at the cell surface), e.g. by serology (complement mediated lymphocytotoxicity assay), or by biochemical analysis (immuno-precipitation and one-dimensional isoelectric

focusing). Moreover, DNA typing can also be performed DNA (molecular level), e.g. by restriction fragment length polymorphism (RFLP), or polymerase chain reaction (PCR) based methods. Serologic methods were one of the first used for routine typing. It is a relatively cheap, quick and reliable method for screening a population for MHC polymorphisms, and, therefore, a powerful tool for MHC - disease association studies.

At the beginning of this research, serology was operational in cattle, but only for detection of class I polymorphisms. Because of the limited knowledge on the genetic organization and polymorphism of the class II region in cattle, the bovine class II serology was in its infancy. Fortunately the techniques used in the bovine class I serology could be applied for the development of bovine class II serology. Furthermore, several techniques routinely used in human serotyping were modified for use in cattle. On this basis, several class II alloantisera were produced by means of allo-immunization (CHAPTER 2).

These alloantisera were analyzed together with alloantisera produced by three other research groups working on class II serology. This resulted in the screening of the largest number of bovine class II alloantisera ever tested in a single laboratory. An analysis of their reaction patterns revealed 18 serum clusters, named Ds01-Ds18. Two main clusters, Ds01 and Ds03, were closely associated with 11 other clusters. The 18 serum clusters were grouped together in such a manner that 17 provisional class II haplotypes could be defined (CHAPTER 3).

The serological definition of class II polymorphism did not appear to be as clear cut as the serotyping of class I. The interpretation of the reaction patterns of the alloantisera was especially difficult. In man, it has been shown that mixtures of antibodies specific for the products of the class II genes, DR and DQ, can complicate the interpretation of the reaction patterns of the class II alloantisera. In the present study the bovine class II alloantisera also contained supposed to be mixtures of different antibody populations. Since both class II genes, DR and DQ, are involved in regulation of the immune response, it was important be able to discriminate between them. Therefore, class II alloantisera were analyzed for their DR and DQ specificities.

To study this aspect, first an absorption study was performed. Subsequently, the monoclonal antibody-specific immobilization of lymphocyte antigen assay (MAILA) was applied. The MAILA assay makes it possible to discriminate between antibodies against the DR and DQ molecules. This test is used on a routine basis in MHC research in man, and was modified for our BoLA class II study. Alloantisera associated with the serum cluster Ds03 were analyzed. The absorption study revealed that the

Ds03 related alloantisera indeed contained several antibody populations. Subsequently, the MAILA-assay indicated that the Ds03 specificity was encoded by DQ, whereas two Ds03 sub-specificities, Ds10 and Ds11 were more closely associated with DR molecules (CHAPTER 4).

Another strategy for analyzing the alloantisera for DR and/or DQ associated reactivity is the comparison of the serological definitions of class II polymorphisms with data obtained by other typing methods. In this study, the results of the class II serology were compared with those obtained by biochemical analysis (1D-IEF) for DR and DQ, and subsequently compared through the results obtained with two molecular typing methods: polymerase chain reaction RFLP for DR (DRB3-PCR RFLP) and RFLP for DQ (DQA and DQB RFLP).

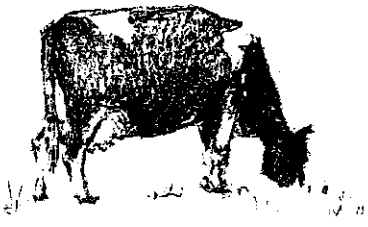
Biochemical analysis of bovine DR polymorphisms was already a routine technique, but biochemical analysis of bovine DQ polymorphism was not operational until modification of the standard 1D-IEF method for DR typing. The comparison between the typing results obtained with both serology and 1D-IEF confirmed the serological definition of haplotypes. It became also apparent that these two methods for the detection of MHC at the gene product level are complementary: one serologically defined class II haplotype can be associated with various 1D-IEF types, and reversely, one 1D-IEF types can be associated with various serologically defined class II haplotypes (CHAPTERS 3 and 5). Also the comparison of class II serology with the two DNA based methods confirmed the serologically defined class II haplotypes. RFLP typing for DQ polymorphism even increased the resolution of class II polymorphism (CHAPTER 6).

Evaluation of all collected typing data revealed a division of the serological defined class II haplotypes in three main groups. This division is probably associated with the DQ polymorphism (CHAPTER 8). Two main groups associated with the clusters Ds01 and Ds03 comprise several subgroups. Association with DR polymorphisms is proven for two subgroups (Ds10, Ds11), for the others the specificity is not known (CHAPTER 4).

The extended typing performed to support the class II polymorphism as defined by serology, resulted in a thorough characterization of bovine class II polymorphism in Holstein Friesians. Furthermore, it provided data on the distribution of MHC polymorphisms in the herd used for this study as well. However, one of the most important aspects is the fact that several animals homozygous for class I and class II were detected (CHAPTER 6). Homozygous animals are indispensable for functional studies on the immune response and the studies as described in CHAPTER 7.

In CHAPTER 7, the reactivity of monoclonal antibodies, recognizing class II molecules in man (anti-HLA mAb) is described. The approach was based upon the hypothesis that sequence homology in the class II region of man and cattle would be sufficient for cross-reaction of these anti-HLA mAb between both species. The data revealed that this was not the case. However, it is clear that the use of well defined non-human class II positive cells may provide additional information on anti-HLA mAb reaction patterns.

In conclusion, detection of bovine class II polymorphisms by serology is possible now. Due to the extensive analysis of the serologically defined class II haplotypes with biochemical and DNA based techniques, further insight was obtained into the reactivity of the class II sera, and distinction by serology between DR and DQ turned out to be feasible. In view of the enormous progress achieved in DNA technology, detection of bovine MHC polymorphisms by class II serology no longer has any direct application for identifying genetic markers in large scale association studies. On the other hand, it is beyond dispute that serological class II typing is very important for functional studies concerning the immune response.



SAMENVATTING

SAMENVATTING

Bij rundvee is variatie waar te nemen in respons op infectieuze agentia, die mede veroorzaakt wordt door de verschillen tussen individuen in genetische resistentie tegen ziekteverwekkers. Het opnemen van genetische resistentie tegen economisch belangrijke ziekten in fokprogramma's zou mogelijk kunnen bijdragen tot een betere beheersing van het voorkomen van infectieuze ziekten. Toepassing van genetische resistentie in de rundveehouderij en/of fokprogramma's vereist de definitie van genetische markers voor ziektegevoeligheid en/of immuun-gemedieerde weerstand. Het major histocompatibility complex (MHC) kan als een goede kandidaat hiervoor beschouwd worden.

Het MHC is een gebied op een chromosoom, waar een aantal zeer polymorfe genen gelokaliseerd zijn. De MHC genen coderen voor de zogenaamde MHC moleculen, die zich op het oppervlakte van cellen bevinden. Deze moleculen zijn van essentieel belang in de immuunrespons, aangezien zij immunogene peptiden binden en aanbieden aan het immuunsysteem. Er zijn twee klassen van MHC moleculen: klasse I, aanwezig op alle kernhoudende cellen, en klasse II, alléén aanwezig op een groep cellen die direct betrokken zijn bij de immuunrespons. De expressie van MHC genen is co-dominant: de produkten van beide homologe chromosomen (zowel de paternaal als de maternaal geërfde genen) van een individu zijn aanwezig op het oppervlakte van de cel. Doordat de MHC genen zo polymorf zijn (per gen veel allelen), heeft ieder individu zijn 'eigen' combinatie van MHC allelen.

Ten tijde van de aanvang van deze studie, waren er bij de mens reeds vele associaties tussen ziektegevoeligheid en het MHC beschreven. Met name karakterisering van de klasse II genen, heeft het aantal publikaties en de significantie van MHC geassocieerde ziekten doen toenemen. In het rund waren ook reeds diverse MHC-ziekte associaties beschreven. Echter, in deze studies was alleen het klasse I polymorfisme gebruikt als marker. De reden hiervoor was dat de kennis over de genetische organisatie en het polymorfisme van het bovine MHC klasse II gebied toen nog zeer beperkt was.

Bovengenoemde aspecten, waren de belangrijkste aanleiding voor de studie beschreven in dit proefschrift: een analyse van het klasse II polymorfisme in het rund. Verbetering van de karakterisering van het bovine MHC klasse II gebied, zal bijdragen tot verdere identificatie van de rol van klasse II in gevoeligheid voor infectie ziekten bij het rund.

Voor detectie (het typeren) van het MHC polymorfisme van individuen zijn verschillende methoden beschikbaar. MHC typering kan plaatsvinden aan de MHC moleculen op het cel-oppervlakte (produkt niveau), bijvoorbeeld door middel van serologie (complement afhankelijke lymfo-cytotoxiciteits test), of door biochemische analyse (immuno-precipitatie en een-dimensionale isoelectrische focusering (1D-IEF). Maar ook kan typering plaatsvinden met behulp van het DNA (moleculair niveau), bijvoorbeeld door middel van restrictie fragment lengte polymorfisme (RFLP), of een methode gebaseerd op de polymerase ketting reactie (PCR). Serologie is een van de eerste methodes ontwikkeld voor het routinematig typeren bij de mens. Het is een goedkope, snelle en betrouwbare methode voor het screenen van een populatie op MHC polymorfisme, en daarom een waardevol gereedschap voor MHC - ziekte associatie studies.

Bij aanvang van deze studie was bij het rund de serologie reeds operationeel, echter alleen voor detectie van het klasse I polymorfisme. Gezien de beperkte kennis over de genetische organisatie en het polymorfisme van het klasse II gebied in het rund, stond ook de bovine klasse II serologie in zijn kinderschoenen. Bij het opstarten van de klasse II serologie kon dus gebruik gemaakt worden van technieken operationeel in de klasse I serologie van het rund. Dit was echter niet voldoende, dus zijn ook nog verscheidene technieken die routinematig gebruikt in de humane klasse II serologie gemodificeerd voor het gebruik in het rund. Hierop gebaseerd zijn met behulp van alloimmunisaties verschillende klasse II alloantisera geproduceerd (HOOFDSTUK 2).

Deze alloantisera zijn vervolgens geanalyseerd, tezamen met de alloantisera van drie andere onderzoeksgroepen. Deze analyse resulteerde in een screening van het grootste aantal bovine klasse II alloantisera ooit getest in één laboratorium. De analyse van de reactie patronen van de klasse II alloantisera resulteerde in 18 serum clusters, welke Ds01-Ds18 genoemd werden. Er waren twee hoofd clusters, Ds01 en Ds03, welke nauw geassocieerd zijn met 11 andere clusters. De 18 serum clusters zijn op zodanige wijze gegroepeerd, dat 17 voorlopige klasse II haplotypen gedefinieerd konden worden (HOOFDSTUK 3).

De serologische definitie van het klasse II polymorfisme bleek niet zo eenduidig als de serologische typering van het klasse I polymorfisme. Met name de interpretatie van de reactie patronen van de alloantisera was moeilijk. Bij de mens is gebleken dat de aanwezigheid van mengsels van verschillende antilichaampopulaties, specifiek voor de produkten van de klasse II genen, DR en DQ, de interpretatie van de reactie patronen van de alloantisera bemoeilijkt. In de onderhavige studie werd er vanuit

gegaan dat ook de bovine klasse II alloantisera mengsels zijn van verschillende antilichaampopulaties. Aangezien beide klasse II genen, DR en DQ, betrokken zijn in de regulatie van de immuunrespons, is het belangrijk om onderscheid te kunnen maken tussen DR en DQ polymorfisme. Zodoende zijn de klasse II alloantisera geanalyseerd voor hun DR en DQ specificiteit.

Eerst werd een absorptiestudie uitgevoerd, vervolgens werd de 'monoklonale antilichaam-specifieke immobilisatie van lymfocyt antigen'(MAILA) test toegepast. Met behulp van de MAILA test is het mogelijk onderscheid te maken tussen antilichamen specifiek voor DR en DQ. Deze test wordt routinematig gebruikt in het MHC onderzoek bij de mens, en is gemodificeerd voor het BoLA klasse II onderzoek. Dit aspect werd nader uitgewerkt met alloantisera geassocieerd met het serumcluster Ds03. Uit de absorptiestudie bleek dat Ds03 gerelateerde alloantisera inderdaad verschillende antilichaampopulaties bevatten. Vervolgens, toonde de MAILA test aan dat specificiteit Ds03 gecodeerd werd door DQ genen, terwijl twee Ds03 subgroepen, Ds10 en Ds11 geassocieerd waren met DR polymorfisme (HOOFDSTUK 4).

Een andere strategie om klasse II alloantisera te analyseren voor DR en/of DQ geassocieerde reactiviteit, is het vergelijken van de serologisch gedefinieerde klasse II typering met die verkregen door het toepassingen van andere typeer methoden. In deze studie zijn de resultaten van de klasse II serologie eerst vergeleken met die van de biochemische analyse (1D-IEF)) voor DR en DQ, en vervolgens met de resultaten verkregen met twee moleculaire typeer methoden: PCR RFLP voor DR (DRB3-PCR RFLP), en RFLP voor DQ (DQA en DQB RFLP).

Biochemische analyse van het bovine DR polymorfisme werd al routinematig gedaan, maar de biochemische analyse van bovine DQ polymorfisme bestond niet. Deze methode werd pas mogelijk na modificatie van de standaard 1D-IEF methode gebruikt voor de DR typering. De vergelijking tussen de typeer resultaten verkregen met serologie en 1D-IEF bevestigde de meeste serologisch gedefinieerde haplotypen, maar tevens bleken deze twee methoden voor detectie van MHC op produkt niveau elkaar aan te vullen: één serologisch gedefinieerde klasse II haplotype kan geassocieerd zijn met verschillende 1D-IEF typen, terwijl één 1D-IEF type geassocieerd kan zijn met meerdere serologisch gedefinieerde klasse II haplotypen (HOOFDSTUK 3 en 5). Ook de vergelijking van de klasse II serologie met de twee DNA gebaseerde methoden bevestigde de serologisch gedefinieerde klasse II haplotypen. De mate van detectie van het klasse II polymorfisme werd zelfs vergroot door de RFLP typering voor DQ polymorfisme (HOOFDSTUK 6).

De evaluatie van alle typeer data betreffende het klasse II polymorfisme, geeft aan dat de serologisch gedefinieerde klasse II haplotypen in drie hoofdgroepen zijn op te

delen. Deze opdeling is zeer waarschijnlijk geassocieerd met DQ polymorfisme (HOOFDSTUK 8). Alleen de twee hoofdgroepen die geassocieerd zijn met de clusters Ds01 en Ds03, hebben meerdere subgroepen. Van enkele subgroepen is bewezen dat ze geassocieerd zijn met DR polymorfisme, van de rest is de specificiteit nog niet duidelijk (HOOFDSTUK 4).

De uitgebreide typering van het bovine klasse II polymorfisme uitgevoerd ter ondersteuning van de serologische definitie van het klasse II polymorfisme gedefineerd met behulp van serologie, heeft geresulteerd in degelijke karakterisering van het bovine klasse II polymorfisme aanwezig in 'Holstein Friesians'. Verder werd het hierdoor mogelijk frequentie gegevens betreffende de distributie van het MHC polymorfisme in de rundvee koppel die voor deze studie gebruikt werd te presenteren. Maar, een van de belangrijkste aspecten is, dat er verscheidene dieren zijn gevonden die homozygoot zijn voor klasse II polymorfisme (HOOFDSTUK 6). Zulke dieren zijn onmisbaar in functionele studies betreffende de immuunrespons en in studies zoals beschreven in HOOFDSTUK 7.

In HOOFDSTUK 7 wordt de reactiviteit van monoclonale antilichamen die reageren met klasse II moleculen van de mens (anti-HLA mAb) beschreven. De hypothese was dat de sequentie homologie in het klasse II gebied bij mens en rund voldoende zou zijn voor kruisreactie door anti-HLA mAb tussen beide species. Dit bleek echter niet zo te zijn. Wel werd duidelijk dat het gebruik van goed gedefinieerde niet-humane klasse II positieve cellen, aanvullende informatie op het reactie patroon van de anti-HLA mAb kan geven.

Geconcludeerd kan worden, dat detectie van tot expressie gebracht bovine klasse II polymorfisme door middel van serologie nu mogelijk is. Dankzij de uitgebreide analyse van de serologisch gedefinieerde klasse II haplotypen met biochemische en DNA gebaseerde technieken, is een goed inzicht verkregen in de reactiviteit van de klasse II sera, en onderscheid met behulp van serologie tussen DR en DQ polymorfisme is nu mogelijk. Gezien de enorme vooruitgang geboekt in de DNA technologie, heeft detectie van MHC polymorfisme bij het rund door middel van klasse II serologie weinig toepassing meer in associatie studies. Daarentegen, blijft de serologische klasse II typering nog wel zeer belangrijk voor functionele studies betreffende de immuunrespons.

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DANKWOORD

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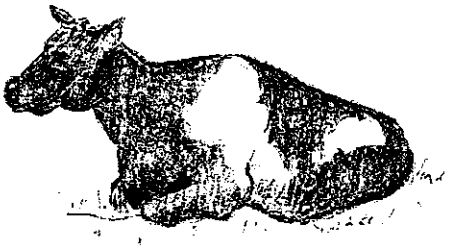
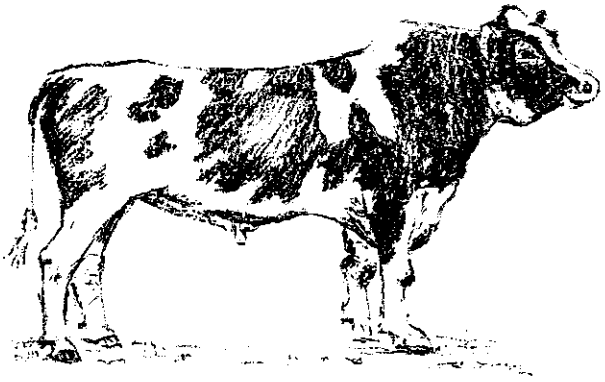


CURRICULUM VITAE

Philomeen Rose Nilsson werd geboren op 4 juni 1959 te 's-Gravenhage. In 1978 behaalde zij het diploma Atheneum B aan de Rijksscholengemeenschap 'J.H. Tromp Meesters' te Steenwijk, en begon zij met de opleiding voor Zoölogisch analist te Amsterdam. Na afronding daarvan, begon zij met de studie Biologie aan de Rijksuniversiteit Utrecht. Het kandidaatsexamen, met specialisatie Medische biologie, werd behaald in 1983. In augustus 1987 studeerde zij af, met als hoofdvak Parasitologie, bijvakken Scheikundige Dierfysiologie en Veterinaire Immunologie, en nevenvak Tropische Diergeneeskunde en Protozoölogie. Voor het onderzoek uitgevoerd in het kader van het hoofdvak Parasitologie ontving de auteur van dit proefschrift in 1986 de prijs 'ex Artikel 60 van de wet op Wetenschappelijk Onderwijs'.

Per 1 oktober 1988 werd zij als assistent-in-opleiding aangesteld bij de vakgroepen Veehouderij, en Experimentele Diermorfologie en Celbiologie aan de Landbouwuniversiteit Wageningen, op een door de Programmacommissie Landbouwbiotechnologie (PcLB) gefinancierd onderzoeksproject. Deze aanstelling heeft geresulteerd in dit proefschrift. Het onderzoek dat beschreven is in dit proefschrift is uitgevoerd in nauwe samenwerking met de vakgroepen Veefokkerij (Landbouwuniversiteit Wageningen), de afdeling Immunologie van de vakgroep Infectieziekten en Immunologie van de Faculteit Diergeneeskunde (Rijksuniversiteit Utrecht) en de afdeling Immunohaematologie en Bloedbank van de Faculteit Geneeskunde (Rijksuniversiteit Leiden).

Vanaf 1 november 1994 is zij aangesteld bij de afdeling Immunologie van de vakgroep Infectieziekten en Immunologie van de Faculteit Diergeneeskunde (Rijksuniversiteit Utrecht).



ADDENDA

Addendum 1. Pedegree information of 79 sires used between 1982 and 1993 in the herd of the 'A.P. Minderhoudhoeve' of the Wageningen Agricultural University: identification numbers of the sires (SIRE), father (SS) and grand-father of the sires (SSS), names of the sires (NAME), and their infered MHC haplotypes.

				HAPLOTYPES OF SIRE	
SIRE	SS	SSS	NAME	(A-DH)	(A-DH)
001	161	110	Frico 2 v.d Kaag	w17-07A	A32----
002	150	102	Trudie's Pan	A16-27A	A05-28A
003	162	103	Sopsum 10	A05-28A	A10-18A
004	153	104	De Beuk	A18----	-------
005	095	085	Arjen	A16----	-------
006	270	091	Himster 539	A11-24A	w17-07A
007	229	096	Hol-stiens T.I.Bold(RF)	NW1-16A	A16----
008	271	087	String Arko 84	A19-24A	A03-12C
009	272	092	Bos Ignatius	A19-24A	A12-18X
010	273	093	Red Triple Puck Sun	-------	A09-03A
011	160	122	Frank 50	-------	w17-07A
012	161	110	Iepenhof 243	A32-07A	-------
013	265	094	Martha's Jemini	-------	A20-08A
014	274	234	Nuboxer Walesa	A14-07A	A19-24A
015	275	095	Panorama 97	A15-22B	A11-24A
016	276	094	Pietje's Pieter	A12-16A	w17-07A
017	273	093	Marco	A19-11A	A20-08A
018	277	093	Vormthoeve 47	A16-16A	A10-03X
019	247	094	Cornelis	A19-24A	A10-26A
020	278	095	Bat	A20-08A	-------
021	274	234	Vechtz.Rocket Alex	A14-07A	A13-23C
022	276	094	Cremerhoeve 1	A12-16A	A14-07A
023	279	089	O.A.Reh ET	w44-07A	A20-08A
024	274	234	Ayolt 2	A14-07A	A16----
025	274	234	Blecma's Takomst 57	A15-22B	A20-08A
026	282	226	Research	P03-32A	A14-07A
027	274	234	Jan de Hurdrider	A15-22B	A19-24A
028	280	082	Botermijn 614 ET	A20-23E	A10-03A
029	274	234	Euro's Rocket 241	A15-22B	A10-26A
030	279	089	Crian 20	A11-12C	A20-08A
031	281	226	Robertus Troy	P03-32A	A19-24A
032	279	089	Hendo 32	-------	A18-22I
033	279	089	Zandvruchter 21	A11-12C	A20-08A
034	282	084	Ivan	A13-23B	-------

035	283	083	Feikje's Enchantment	A10-26A	A15-22B
036	275	095	Klaas 7	A31-28A	A20----
037	284	247	Miens Arko 2	A19-24A	A11-24A
038	285	271	Research 2	A18-22I	--------
041	290	094	Bear Path Fantastic	A20-08A	--------
042	271	087	Steiger	A19-24A	A11-24A
043	290	094	Valentin	A13-23E	A14-27A
044	273	093	F.G. Acres Felix	A19-11A	A11-24A
045	286	081	Gryphus Bea Sheik	A10-03A	A19-24A
046	289	087	HazelBush Rockm. Dictator	A20-08A	A20-08A
047	290	094	Thonyma Vagabond	A13-23E	A12-16A
048	279	089	Snells-Bush Sens Hans	A11-12C	A11-24A
049	291	084	Plushanski Spike	A14-11A	A20-08A
050	290	094	Bos Immo	A12-16A	A10-03C
051	287	094	Budjon Chaser	A19-24A	A31-11C
052	290	094	Volker	A12-16A	A06-10C
053	288	095	Freebrook Sexation Amos	A20-08A	A14-11A
054	279	089	Skalsumer Star	w44-07A	A20-08A
055	274	234	Bockema Rocket 2	A14-07A	A19-24A
056	095	085	Laura Le Elevation Magic	A20-08A	A14-11A
057	281	226	Trokar	P03-32A	A15-22H
058	280	082	Thonyma Gambler	A20-08A	A12-16A
059	288	095	Paltzer Sexation Bert	A20-08A	A12-24A
060	292	095	Bareck	A20-08A	A15-22B
061	290	094	Keegster Martijn	A13-23D	A16-27A
062	293	094	Shl Chief Elite	A19-24A	A14-11A
063	304	094	Conant-AS Frozen Boy	A15-22B	A20-08A
064	303	094	Arend	A15-22X	--------
065	275	095	Betje's Very 2	A31-28X	A19-24X
066	290	094	Volant	A12-16X	A05-28X
067	303	094	Nino van ter Kuile	A15-22X	A19----
068	290	094	Vers	A13-23X	A15-22X
069	305	271	Botermijn 647	A14----	A10-03X
070	307	290	Plushanski Yuma	A12-16X	A20-08X
071	290	094	Oudenhoef 176	A12-16X	A31-28X
072	279	089	Groenlander 116	A11-12X	A16-27X
073	089	055	Riehlholm Mutual	w44-07X	A15-22X
074	290	094	Feikje's Boppe	A13-23X	A15-22X
075	306	290	Etazon Economy	A12-16X	A13-23X
076	306	290	Frankenhof 21	A12-16X	A15-22X
077	269	055	Delta Boy Galazon	w44-07X	A20-08X
078	303	094	Feikje's Wijnsteker	A15-22X	A15-22X
079	269	055	Coba's Ned boy	A32-22X	A14-22X
080	303	094	Feikje's Jetspot	A19-24X	A15-22X
990	303	094	Skalsumer Sunny Boy	A15-22X	A20-08X

X Class II DQA and DQB type not known

-- Class I and class II type not known

Addendum 2. Pedegree information of cows of the herd of the 'ir. A.P. Minderhoudhoeve' of the Wageningen Agricultural University.
The cows are members of 46 families. The family trees are given on the supplementary sheet.
The cows are listed as followed: 1 2
3

1. Identification number of the cow, e.g. A2617
2. Identification number of the sire of which the first/second MHC haplotype of the cow is inherited, e.g. 072B/036A
3. First/second MHC haplotype of the cow, e.g. A16-27X/A31-28A

The death of those we love is the painful, daily reminder of the frailty of our lives.