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Transfection into mouse L cells of genes encoding two serologically and functionally distinct bovine class I MHC molecules from a MHC-homozygous animal: evidence for a second class I locus in cattle

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SUMMARY

w10 and KN104 are distinct class I major histocompatibility complex (MHC) serological specificities present in Boran (*Bos indicus*) cattle. Although these specificities are commonly expressed together, they may also be expressed independently. To establish whether w10 and KN104, when expressed together, are on the same or different molecules, and whether a second class I MHC locus exists in cattle, genomic DNA from an animal homozygous for a haplotype encoding the w10 and KN104 specificities was transfected into thymidine kinase-deficient mouse L cells (Ltk$^-$ cells), and the transfected cells were screened with monoclonal antibodies (mAb) specific for the w10 or KN104 allospecificities. Two different populations of transfectants were identified: the cells of one population reacted only with w10-specific mAb, whereas those of the other population were recognized only by the KN104-specific mAb. Alloreactive cytotoxic T lymphocytes (CTL) also distinguished between the two populations. Two CTL clones, shown to be restricted by the KN104 specificity, killed only those L cells expressing molecules recognized by the KN104-reactive mAb. Of eight CTL clones which recognized class I molecules associated with the w10 specificity, four killed the L cells expressing the w10 specificity. The remaining four clones did not kill either population of transfectants. Finally, immunoprecipitation studies revealed that both populations express full-length bovine class I MHC molecules. These results demonstrate that the w10 and KN104 specificities are on distinct class I molecules. As the genes encoding these molecules were derived from a MHC-homozygous animal, the findings also provide strong evidence that there are at least two classical class I loci in cattle.

INTRODUCTION

Cytotoxic T lymphocytes (CTL) recognize foreign antigens presented on cell surfaces in association with self-major histocompatibility complex (MHC) glycoproteins, a phenomenon known as MHC restriction (Zinkernagel & Doherty, 1979). In each of the two most extensively studied species, man and mouse, there are three genetic loci encoding functional class I MHC molecules involved in CTL recognition (Steinmetz & Hood, 1983).

Abbreviations: complete DMEM, Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, gentamycin at 50 μg/ml and 50 μM 2-mercaptoethanol; CTL, cytotoxic T lymphocyte; FACS, fluorescence-activated cell sorter; Ltk$^-$, thymidine kinase-deficient mouse L cells; mAb, monoclonal antibody/antibodies; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline, pH 7·4; TCGF, T-cell growth factor.

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MHC restriction of bovine CTL recognition has also been demonstrated (Goddeeris *et al.*, 1986), confirming the importance of class I MHC molecules in cattle. At present, 33 bovine class I specificities have been defined serologically in international workshops (Bull *et al.*, 1986). There is no conclusive evidence that these specificities are encoded at more than one locus, although some serological (Stear, Newman & Nicholas, 1982) and biochemical (Bensaid *et al.*, 1988) evidence has been presented which suggests the expression of a second locus. Determination of the number of expressed class I MHC loci is important for a more complete understanding of the role of MHC molecules in restricting bovine CTL responses.

Recent studies of a particular MHC haplotype in Boran (*Bos indicus*) cattle have established that the products of this haplotype display two distinct serological class I specificities, called w10 and KN104 (Kemp, Spooner & Teale, 1988). The functional importance of the KN104 specificity has been demonstrated in the studies in which CTL from cattle to infection by the protozoan parasite, *Theileria parva*, were restricted by molecules carrying this specificity (Goddeeris *et al.*,

1986). The w10 and KN104 specificities are defined by alloantisera and monoclonal antibodies (mAb): mAb IL-A10 and IL-A34 identify the w10 specificity, whereas mAb IL-A4 identifies the KN104 specificity. In the population of *B. indicus* cattle studied, these specificities are usually expressed together. However, in a small proportion of animals, only one or other specificity is expressed (Kemp *et al.*, 1988). This prompted the question of whether these specificities, when expressed together, are present on the same or different molecules.

To answer this question, genomic DNA from an animal known to be homozygous for MHC haplotype encoding the w10 and KN104 specificities was transfected into mouse fibroblasts. Transfectants expressing bovine class I MHC antigens were selected, and three cloned transfected cell lines were analysed with mAb and alloreactive CTL specific for the w10 and KN104 specificities.

MATERIALS AND METHODS

Monoclonal antibodies

The following ILRAD mAb were used in the study. IL-A88 (IgG2a) reacts with a monomorphic determinant on bovine class I MHC molecules (A. Bensaid and S. P. Morzaria, unpublished data); IL-A10 (IgM) and IL-A34 (IgG2a) react with class I determinants associated with the w10 specificity (A. J. Teale, W. I. Morrison, S. J. Kemp and A. Bensaid, unpublished data); IL-A4 (IgM) reacts with the KN104 specificity (Kemp *et al.*, 1988); IL-A7 (IgG2a) reacts with a polymorphic class I determinant present on cells from all $w10^+$ animals and some $w10^-$ animals (Bensaid *et al.*, 1988); IL-A21 (IgG2a) reacts with a monomorphic determinant on bovine class II MHC molecules (J. Ellis, unpublished data); IL-A11 (IgG2a) and IL-A17 (IgG1) react with the bovine homologues of CD4 and CD8, respectively (Baldwin *et al.*, 1986; Ellis *et al.*, 1986). None of the class I-specific mAb recognizes murine class I antigens expressed by L cells.

Preparation of genomic DNA

The animal selected as donor of genomic DNA for transfection was a Boran female, E98. This animal was produced by mating a bull of the class I MHC phenotype w10/w13 with a half-sister of class I phenotype w4/w10. The sire and dam had inherited the same w10 haplotype from their common parent. The homozygosity of E98 (class I phenotype w10) was confirmed by serotyping, mixed leucocyte reactions, field inversion gel electrophoresis analysis with MHC class I and class II probes and determination of gene dosage by fluorescence-activated cell sorter (FACS) analysis with mAb specific for the gene products (A. J. Teale and A. Bensaid, unpublished data).

Peripheral blood mononuclear cells (PBMC) were obtained from E98 by centrifugation of blood through Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) (Lalor *et al.*, 1986), and genomic DNA was prepared from the PBMC by a modification of a method described previously (Alberti & Herzenberg, 1988). About $1–2 \times 10^8$ PBMC were lysed by suspending them at 10^7 cells/ml in 4 M guanidinium isothiocyanate and rocking gently at room temperature for 4 hr. The cell lysate was layered over 5 ml 5·7 M cesium chloride in polyallomer tubes and centrifuged in a SW-41 rotor (Beckman, Fullerton, CA) at 154,000 *g* for 20 hr at 20°. The viscous DNA layer just below the interface was recovered, and dialysed three times against 100 volumes of 10 mmol Tris, 1 mmol EDTA, for 24 hr at 4°. In a modification of the previously described method (Alberti & Herzenberg, 1988), the DNA was not treated with proteinase K nor RNAse A, nor extracted with phenol/chloroform. The omission of these treatments appeared not to affect transfection efficiency. The DNA was precipitated with ethanol and was finally suspended in 1·0 ml sterile, distilled water.

Thymidine kinase-deficient mouse L cells (Ltk^- cells) were routinely cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, gentamycin at 50 μg/ml and 50 μmol 2-mercaptoethanol (complete DMEM). For transfection, cells were plated at about 5×10^5 cells per 100 mm tissue culture dish ('Lux'; Miles Laboratories, Naperville, IL) 24 hr prior to use.

Transfection

Genomic DNA was transfected into Ltk^- cells by calcium phosphate-mediated co-transfection (Kavathas & Herzenberg, 1986) with a selectable plasmid containing the chicken thymidine kinase gene (Perucho *et al.*, 1984). Following transfection, the cells were placed in complete DMEM for 24 hr before transfectants were selected in complete DMEM containing hypoxanthine, aminopterin and thymidine.

Selection of positive transfectants

When colonies of transfected cells were visible macroscopically (usually at about 10–12 days after transfection), the positive transfectants were selected by FACS analysis. All colonies were removed with phosphate-buffered saline (PBS), pH 7·4, Ca^{2+}/Mg^{2+}-free and containing 0·6 mmol EDTA, and the cells were pooled and incubated in the appropriate mAb in the form of ascitic fluid diluted in PBS to a suitable working concentration. After washing, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Southern Biotechnology, Birmingham, AL), and sorted using a FACS ('FACStar Plus'; Becton-Dickinson, Sunnyvale, CA).

The transfected cells were sorted initially with mAb IL-A88 or mAb IL-A7. On the first round of sorting, the positive cells were selected 'blind', in that a distinct, positive population was not always apparent. In this case, a population comprising the most brightly fluorescing cells (about 0·5% total cell population) was selected and further cultured until sufficient cells were obtained to be sorted again with the class I-specific mAb. If a positive population was apparent, it was usually seen as a discrete, brightly stained group of cells. After one or two further enrichments using the FACS to obtain a population consisting of more than 90% positive transfectants, the cells were cloned by limiting dilution.

Immunoprecipitation

Surface molecules of transfected and untransfected L cells, and PBMC from E98, were radioiodinated and immunoprecipitated by methods described previously (Bensaid *et al.*, 1988). Each lysate was precleared with an irrelevant mAb (IgG2a) and protein A–Sepharose (Pharmacia, Uppsala, Sweden), and immunoprecipitated with the IgG2a myeloma protein, UPC-10 (Sigma, St Louis, MO), and protein A–Sepharose. After centrifugation, protein A–Sepharose alone was added to remove any remaining antibody from the supernatant and, after further centrifugation, the bovine class I molecules were immunoprecipitated with mAb IL-A88 and protein A–Sephar-

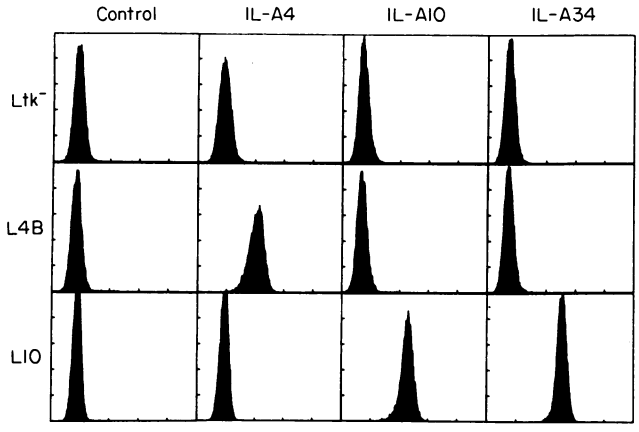

Figure 1. FACS analysis of transfected and untransfected cell lines. The transfected cells were cloned from populations selected as positive to mAb IL-A88 from independent transfections. The cells were stained with mAb IL-A4, IL-A10, IL-A34 or IgG2a and IgM isotype control antibodies.

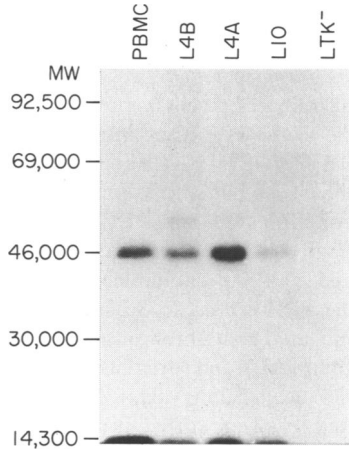

Figure 2. Immunoprecipitation analysis of PBMC from animal E98 (PBMC), untransfected Ltk⁻ cells (LTK⁻) and transfected cells reactive with mAb IL-A4 (L4A and L4B) or mAb IL-A10 (L10). The cells were labelled with radioactive iodine and the bovine MHC molecules precipitated with mAb IL-A88. The precipitates were analysed on a 10% polyacrylamide gel, followed by autoradiography. The numbers on the left indicate the position and MW of a ^{14}C-methylated protein mixture used as markers.

ose. The precipitates were analysed under reducing conditions by SDS–PAGE through 10% (w/v) acrylamide gels, followed by autoradiography. No labelled proteins were observed to be precipitated with UPC-10 (results not shown). A mixture of ^{14}C-methylated proteins (Amersham, Amersham, Bucks, U.K.) was used to indicate the relative molecular weight (MW) of the precipitated proteins.

Derivation of CTL clones

Cloned, alloreactive CTL lines specific for class I MHC antigens of animal E98 were generated from animal E192, which has the W6/-class I MHC phenotype. PBMC from animal E192 were stimulated with E98 PBMC in an allogeneic mixed leucocyte reaction as described previously (Teale *et al.*, 1986). The cells were restimulated 7 days later, and after a further 6 days they were tested for cytotoxic activity; at an E:T ratio of 20:1, they killed 91% of E98 target cells and only 7% of an unrelated target cell population. To enrich for class I-restricted CTL, the cultured lymphocytes were depleted of CD4$^+$ lymphocytes by complement-mediated lysis (Teale *et al.*, 1985) with the CD4-specific mAb, IL-A11. This resulted in killing of almost 90% of the cells. The surviving cells were incubated overnight in medium supplemented with 5% T-cell growth factor (TCGF). The TCGF consisted of conditioned medium from bovine PBMC stimulated with concanavalin A (Teale *et al.*, 1985).

On the following day, cultures were established to obtain cloned T-cell populations, as described previously for *Theileria*-specific T-cell clones (Goddeeris *et al.*, 1986). Briefly, the cells were distributed into round-bottomed microculture plates at 0·3, 1·0 and 3·0 cells per well in the presence of irradiated stimulator and filler cells in medium containing 15% TCGF. The stimulator cells were from a *Theileria*-infected cell line derived from animal D409, which carries an identical class I MHC haplotype to animal E98. After 10 days, the cultures were screened microscopically and from those wells in which there was obvious cell growth, about half of the cells were removed and tested for cytotoxic activity. Cultures showing cytotoxic activity were restimulated and expanded as described previously (Goddeeris *et al.*, 1986).

All cultures utilized RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mmol L-glutamine, 50 μg/ml gentamycin and 50 μmol 2-mercaptoethanol.

Cytotoxicity assays

T-cell clones were initially screened for cytotoxic activity using a 4-hr ^{111}In release assay (Goddeeris *et al.*, 1986). Subsequently they were tested in a standard 4-hr ^{51}Cr-release assay. *Theileria*-infected cell lines derived from Boran cattle of different MHC phenotypes were used as target cells for establishing the MHC specificity of the clones. Selected T lymphoblast cell lines maintained with bovine TCGF were also used as target cells. Several mAb specific for bovine class I MHC antigens were tested for their capacity to inhibit cytotoxicity, by preincubating them with target cells at room temperature for 30 min before adding the effector cells (Goddeeris *et al.*, 1986).

RESULTS

Transfection experiments

Following transfection of genomic DNA from animal E98 into Ltk⁻ cells, the total pool of transfected cells was sorted by FACS with mAb IL-A88 or mAb IL-A7. The selected cells were cloned, and individual cloned lines were analysed with mAb IL-A10, IL-A34 and IL-A4.

In the first transfection experiment, cells reactive with IL-A10 and IL-A34, but not IL-A4, were obtained. From each of two subsequent transfections, cloned cells reactive with IL-A4, but not IL-A10 or IL-A34, were obtained. Figure 1 shows the results of FACS analysis of two such cloned lines, one (L10) from the IL-A10/IL-A34-positive population and one (L4B) from an IL-A4-positive population, together with untransfected (Ltk⁻) cells.

Table 1. Cytotoxic activity* of alloreactive CTL clones for bovine cells of different MHC phenotype and for Ltk$^-$ cells tranfected with bovine class I MHC genes

			Specificity of killing by CTL clones†									
	Phenotype		I		II	III			IV			
Target	w10	KN104	A	B	C	D	E	F	G	H	I	J
E98	+	+	+	+	+	+	+	+	+	+	+	+
F104	+	+	+	+	+	+	ND	+	ND	+	+	+
D540	+	+	ND	+	+	+	ND	+	ND	+	+	ND
C234	+	+	ND	+	+	+	ND	+	ND	ND	+	ND
C447	+	+	ND	+	+	+	+	+	+	ND	+	+
C887	+	+	ND	+	+	+	+	+	+	+	+	+
D85		+	+	+	−	−	−	−	−	−	−	−
K96		+	+	+	ND	−	ND	−	ND	−	−	−
K104		+	ND	+	−	−	ND	−	ND	ND	−	ND
B734	+		−	−	+	−	−	−	−	−	−	−
C538	+		−	−	+	−	−	−	−	−	−	−
B881	+		ND	ND	+	ND	ND	ND	ND	−	−	ND
D232			−	−	−	−	−	−	−	−	−	−
C196			−	−	−	−	−	−	−	−	−	−
Ltk$^-$			−	−	−	−	−	−	−	−	−	−
L10	+		−	−	−	−	−	−	+	+	+	+
L4A		+	+	+	−	−	−	−	−	−	−	−
L4B		+	+	+	−	−	−	−	−	−	−	−

* All assays were performed at E:T ratios ranging from 4:1 to 0·25:1.

† I–IV represent groups of clones of similar specificity. A positive result indicates >60% cytotoxicity, while a negative result represents <7%.

ND, not done.

Immunoprecipitation experiments

In order to compare the bovine MHC molecules expressed by the transfected cells with those expressed on cells from E98, bovine MHC molecules were immunoprecipitated from these cells with mAb IL-A88, and the MW of the molecules was determined by SDS–PAGE and autoradiography. The autoradiograph reproduced in Fig. 2 demonstrates that both the IL-A10/IL-A34-positive and the IL-A4-positive transfected cells express a bovine class I MHC product equal in MW (about 45,000) to that expressed by E98 lymphocytes. The differences in intensity of the bands derived from the transfected cell populations may be due to a variation in the level of expression of the transfected bovine genes or of the iodination of the gene products. The band present at the bottom of the autoradiograph is probably β_2-microglobulin which was co-precipitated with the MHC molecules.

Studies with CTL clones

Ten alloreactive, cloned CTL lines were established from the peripheral blood of animal E192. Nine clones had the CD4$^-$CD8$^+$ phenotype, whilst one was CD4$^-$CD8$^-$ (clone H, Table 1), as defined by reactivity with the anti-CD4 mAb IL-A11 and the anti-CD8 mAb IL-A17. To establish the specificity of the cloned cells, each clone was tested on at least three target cells expressing both the w10 and KN104 specificities, at least two target cells expressing only w10 or KN104 and at least two target cells which expressed neither of these specificities. The results (Table 1) revealed three distinct patterns of killing. Clones A and B killed all target cells expressing the KN104 specificity, clone C killed all bovine target cells expressing the w10 specificity, and the remaining seven clones killed only those bovine target cells expressing both the w10 and the KN104 specificities.

The capacity of mAb IL-A10, IL-A34 and IL-A4, along with appropriate isotype controls, to inhibit cytotoxicity of the CTL clones was tested using a target cell line from the animal F104. This animal is homozygous for the same MHC haplotype as that in E98. Examples of these results are shown in Fig. 3. mAb IL-A4 specifically inhibited, by over 50%, the cytotoxicity of the two clones which recognized all KN104$^+$ target cells, but had no effect on killing by the remaining eight clones. Conversely, the cytotoxicity of the clone which killed all w10$^+$ target cells and six of the seven clones which killed only w10$^+$ KN104$^+$ targets was inhibited by over 80% by mAb IL-A34 and to a lesser extent by mAb IL-A10. These two mAb had no inhibitory effect on the two clones which killed KN104$^+$ target cells. These results indicate that two of the clones are specific for the class I MHC molecule bearing the KN104 serological specificity, and at least seven of the remaining eight are specific for the molecule bearing the w10 specificity.

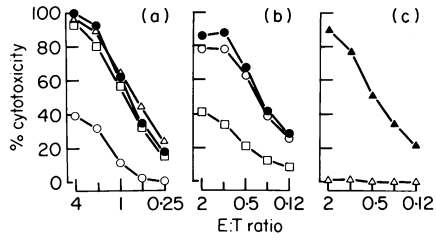

Figure 3. Inhibition of killing of F104 target cells (w10; KN104 homozygous) by alloreactive CTL clones using various mAb. (a) Shows the effect of preincubation with mAb IL-A4, IL-A10 and IL-A34 on the cytotoxic activity of the KN104-specific clone B. (b) and (c) show the effect of preincubation with mAb IL-A4, IL-A10, IL-A34 and an irrelevant antibody, IL-A21, on the cytotoxic activity of the w10-specific clone I. In both cases, the mAb are represented as follows: IL-A4 (○); IL-A10 (□); IL-A34 (△); IL-A21 (▲). Results for assays where no mAb was used are indicated as (●).

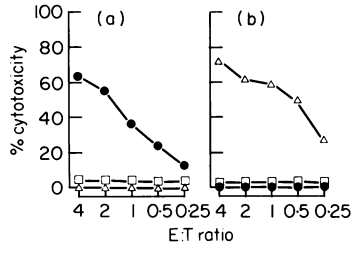

Figure 4. Killing of transfected Ltk$^-$ cells by alloreactive CTL clones. Transfectants carrying the KN104 (a) or the w10 (b) specificity were tested for susceptibility to killing by the KN104-specific clone B (●) or the w10-specific clones F (□) and I (△), at various E:T ratios as indicated. No killing was observed when the clones were tested on the parent Ltk$^-$ cell line.

When the clones were tested on the transfected Ltk$^-$ cells, the two KN104-specific clones (A and B) were found to kill the L4A and L4B transfectants but not the L10 transfectant or the parent Ltk$^-$ cells (Table 1). Four of the remaining eight clones (G, H, I and J) killed the L10 transfectant but did not kill the L4A orL4B transfectants or the parent Ltk$^-$ cells. All four of these clones were from the group which only killed target cells expressing both the w10 and KN104 specificities, and included the one clone (clone J) for which cytotoxicity was not inhibited by mAb IL-A4, IL-A10 or IL-A34. Clone C, which killed bovine cells expressing w10 in the absence of KN104, did not kill the transfectant. The cytotoxic activity of three representative clones, (B, F and I), at various E:T ratios, is shown in Fig. 4.

DISCUSSION

The results presented here indicate that the MHC haplotype studied encodes two distinct class I molecules. This conclusion is based on several differences observed between populations of L cells transfected with class I MHC genes from this haplotype. Firstly, mAb specific for one or other of two serologically defined class I specificities present on this haplotype reacted with the transfected cell populations in a mutually exclusive manner. Secondly, alloreactive CTL clones which were shown to be specific for one or other of these specificities killed the transfected cell populations in the same mutually exclusive manner. The specificity of these clones was established by testing against a panel of semi-matched and mismatched bovine target cells, and by inhibition of the killing of these targets by the mAb specific for the two specificities. The recognition of the transfected cell populations by the alloreactive CTL clones was also specifically inhibited by these mAb (results not shown). Thirdly, immunoprecipitation studies revealed that all transfected cell populations produced full-length bovine class I molecules. Taken together, these results clearly indicate that the transfected cells express two different bovine class I molecules.

Given that the transfected genes were derived from a MHC-homozygous animal, the expression of two different class I molecules could be explained as due to differential processing of a single gene, or to the expression of two class I loci. Mechanisms which could result in the appearance of different products from the same gene include alternative splicing of RNA and variation in glycosylation of the same gene product. Alternative RNA splicing has been described for human (Krangel, 1986) and murine (Transy, Lalanne & Kourilsky, 1984; Lalanne *et al.*, 1985) class I genes. In these studies, both the canonical and non-canonical transcripts were found in the same cell populations. However, if alternative RNA splicing results in the differences seen here between the transfected cell populations, it would imply that each spliced RNA form is produced in one population but not the other, at least within the limits of detection by mAb and alloreactive CTL clones. This appears unlikely, given the results of the experiments mentioned above and the fact that the transfectants were all derived from the same parent cell line and thus might be expected to have similar RNA splicing mechanisms.

Similarly, it is difficult to account for the differences observed between the transfected cell populations in terms of variation in glycosylation of the same gene product in cells derived from the same parent cell line. In addition, this explanation implies that at least some of the CTL clones which discriminate between the transfectants are directed against carbohydrate determinants, and it is yet to be generally established that T lymphocytes can recognize carbohydrate epitopes. Finally, both alternative RNA splicing and glycosylation differences could be expected to result in a difference in the MW of the bovine class I molecules expressed in the different transfected cell populations, which was not observed. Thus, although they cannot be formally excluded, alternative RNA splicing and variation in glycosylation are considered to be unlikely to account for the differences between the transfected cell populations. Rather, these differences are more probably due to the expression of genes from two different class I loci.

Several earlier experiments had suggested the existence of a second class I MHC locus in cattle. Sequential immunoprecipitation of the class I molecules from cells of a heterozygous (w10/KN18) animal with alloantisera and mAb indicated that there were at least five class I molecules on the cells of this animal (Bensaid *et al.*, 1988). However, as these experiments do not preclude the possibilities that the different molecules are post-transcriptionally modified products of a single locus, or that the antibodies used vary in the ability to clear a cell lysate of their respective antigens, the results are inconclusive.

Studies in which alloantisera were used to detect the segregation of serological specificities in related cattle and for co-capping of lymphocytes (Stear *et al.*, 1982) were also interpreted to suggest the expression of a second class I locus. However, no conclusive evidence was presented that the antisera were directed against functional class I MHC products, and the

value of the co-capping experiments is difficult to assess as no data were presented.

Recently, cDNA copies of genes encoding class I molecules were isolated from a library constructed from a bovine B-cell line (Ennis, Jackson & Parham, 1988), and the sequences of two clones encoding different predicted proteins were compared. The authors suggested that the low level of homology between the 3′ untranslated regions of the two sequences indicated that the clones were derived from different loci. However, as the class I phenotype of the cell line has not been established, it is difficult to know whether either or both genes encode functional class I MHC molecules expressed on the surface of bovine cells.

In the course of the present studies, clear differences were observed in the patterns of killing by the CTL clones specific for cells expressing the w10 specificity. Firstly, only one clone, clone C, killed all bovine cells expressing the w10 specificity, but not the L10 transfectant. The remaining seven clones killed only those bovine cells which expressed both the w10 and the KN104 specificities. Secondly, only four of the w10-specific clones killed the transfected cells expressing the w10 specificity. In other words, the w10-specific clones were able to detect differences in serologically indistinguishable w10-bearing molecules. That all of these clones were directed against the w10-bearing molecule, and not the product of a third class I MHC locus, was indicated by the ability of the anti-w10 mAb, IL-A34, to block the cytotoxic activity of seven of the eight clones, and the ability of the remaining clone to kill the L10 transfectant.

An explanation for these observations can be derived from recent experiments which have described two types of variation in serologically identical class I molecules. Firstly, there may be slight structural variations in these molecules which affect the epitopes recognized by CTL, but not by antibodies. Such structural variants have been shown for two human class I antigens, in which differences have been detected by human (Biddison *et al.*, 1980) and murine (Yannelli, Moore & Engelhard, 1985) CTL. For one of these antigens, HLA-A2, other studies (Krangel, Biddison & Strominger, 1983) demonstrated that the structural variations involved amino acid substitutions in a region of the molecule considered important in establishing contact with the corresponding CTL receptor (Bjorkman *et al.*, 1987). The second type of variation centres on the possibility that alloreactive CTL do not recognize the native class I glycoprotein, but react with an endogenously derived self-antigen presented by the class I molecule in a manner similar to the presentation of foreign antigens. Evidence for this possibility comes from recent experiments involving transgenic mice carrying the HLA-A2 gene (Bernhard *et al.*, 1988). Although these mice were tolerant for syngeneic mouse cells expressing the HLA-A2 antigen, they could be induced to produce CTL specific for this antigen when expressed on human cells. The authors concluded that the murine and human versions of HLA-A2 are different, perhaps because they are associated with different endogenously derived molecules which contribute to the epitope recognized by the CTL.

In the present studies, the spectrum of killing of w10$^+$ target cells by clone C compared to the other w10-specific clones may be the result of either mechanism. On the one hand, these differences may be due to a variation in the self-antigens available for presentation by the w10-bearing molecules in the different target cells. However, this implies that clones D–J are specific for self-antigens present in the w10$^+$KN104$^+$ bovine cells but not in the w10$^+$KN104$^-$ bovine cells. A more appealing interpretation is that the difference in the killing of the bovine cells is due to structural variation in the w10-bearing molecules. In this case, one variant is expressed by the w10$^+$KN104$^+$ cells and another by the w10$^+$KN104$^-$ cells. Clone C recognizes both forms of the molecule but clones D-J recognize the variant present only in those target cells which express both w10 and KN104. It should be pointed out that the inability of clone C to kill the L10 transfectant cannot be explained by this hypothesis and is more likely due to different self-antigens in bovine and murine cells. Thus, it appears that both mechanisms which may result in variation in CTL recognition of serologically identical class I molecules could be operating in this situation.

The second difference was detected among the seven clones which showed similar recognition of bovine cells but which could be distinguished by their abilities to kill the transfected cells expressing the w10 specificity. Here, structural variation appears to be an unlikely explanation as all seven clones recognized the w10-bearing molecule expressed by the E98 PBMC, the DNA of which was used to produce the transfectants. If these CTL are detecting differences in self-antigens presented by the w10-bearing molecules, then the clones which killed the w10$^+$KN104$^+$ bovine cells but not the transfected cells must recognize a bovine-specific determinant. The intriguing possibility exists that this determinant is part of the KN104-bearing molecule. That this could occur is suggested by other experiments which have established that alloreactive CTL clones can recognize peptide fragments of one murine class I molecule presented in association with another class I molecule (Song *et al.*, 1988; Olson *et al.*, 1989). Current experiments in this laboratory are examining whether a similar situation occurs with these bovine alloreactive CTL clones.

The results presented here demonstrate the feasibility of using transfection techniques to help to define the number of expressed class I MHC genes. Additionally, transfected cells are valuable tools for producing new serological reagents to MHC molecules, and for studying the role of individual MHC molecules in the restriction of CTL recognition of foreign antigens.

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