A bovine monoclonal antibody detecting a class I BoLA antigen

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Summary. The bovine IgG₁ monoclonal antibody (mAb) ILA70 was made by immunizing a calf with peripheral blood mononuclear cells (PBM) from a BoLA-w10 homozygous heifer and subsequently fusing lymphocytes from the local lymph-node with the heterohybridoma 53B3. Family and population studies, antibody binding inhibition and immunoprecipitation of the target antigen all indicate that ILA70 detects a polymorphic epitope on a bovine class I major histocompatibility complex (MHC) molecule. The antibody is complement fixing and so may be used in a standard cytotoxicity assay. Ascitic fluid with antibody activity many times greater than that of the tissue-culture supernatant has been prepared in nude mice. The antibody-producing heterohybridoma has been subcloned three times and appears to be stable. Such heterohybridomas may prove to be a valuable source of particularly discriminating and informative mAbs for the serological analysis of the products of the bovine MHC.

Keywords: monoclonal antibody, bovine, hybridoma, BoLA, MHC

Introduction

Monoclonal antibodies (mAbs) have a number of advantages over alloantisera for the purpose of analysing the bovine major histocompatibility complex (MHC) (which is expressed as the bovine lymphocyte antigen (BoLA) system). Perhaps the most important of these is that they are available in unlimited quantity, which allows them to be used as standardized reagents in different laboratories both for routine typing and for biochemical studies. However, in our experience, many mouse antibodies are of limited value for many aspects of such studies because they are directed against non-polymorphic epitopes on BoLA molecules. This appears to be particularly true of those detecting BoLA class II antigens.

In studies of the human lymphocyte antigen (HLA) system, a great deal of effort has been expended in making murine mAbs suitable for tissue-typing class II products. The resultant reagents generally detect epitopes which are common to many antigens so that large numbers of such reagents are needed to ‘type’ reliably for.

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class II (Bodmer et al. 1984). In an attempt to overcome this problem, efforts have
been made to make human-anti-human mAbs and a number of such reagents now
exist (reviewed by James & Bell 1987). One particularly successful approach has
been the use of Epstein-Barr virus (EBV) to transform human immune B-cells from
which antibody-secretors can be selected, cloned and expanded and subsequently
fused with an appropriate fusion partner.

There have been several reports of bovine mAbs produced by fusing aminopterin-sensitive mouse-cattle heterohybridomas with lymphocytes from immunized
cattle. These include antibodies directed against bovine red cell antigens (Tucker et
af. 1987), the pilus antigen of *Escherichia coli* (Anderson et al. 1987), testosterone
(Groves et al. 1987) and respiratory syncytial virus (Kennedy et al. 1988). We have
used a similar approach in an attempt to generate a monoclonal reagent directed
against a bovine MHC antigen in order to improve the level of definition of the
products of a specific MHC haplotype beyond that presently possible with mouse
mAbs.

**Materials and methods**

**Immunization.** A Boran (*Bos indicus*) steer, of class I BoLA phenotype w7/w8, was
immunized on three occasions with approximately 3 × 10⁸ PBM obtained from an
inbred Boran heifer which is homozygous for the w10 BoLA specificity. Both w10
haplotypes are known to originate from a single haplotype which was carried by the
grand-sire of this animal. Half of the cells were inoculated subcutaneously and half
intramuscularly in 5 ml of Dulbecco’s phosphate buffered saline (PBS) in the neck
region. A similar immunization was made 8 weeks later. After a further 4 weeks, 3 × 10⁹
PBM in Freund’s incomplete adjuvant were injected into the right gastrocnemius
muscle.

**Fusion.** Four days after the final immunization, the right popliteal lymph node
was removed from the immunized animal under general anaesthesia. The node was
cut into small pieces in RPMI-1640 medium with 20mM HEPES (Gibco, UK),
supplemented with 20% horse serum (Sera-Lab, UK), L-glutamine (2mM),
gentamycin (50μg/ml) and 2-mercaptoethanol (50μM) (growth medium) and cells
were extracted by repeatedly pipetting growth medium over the pieces of tissue. The
growth medium with suspended cells was transferred to a 50-ml polypropylene tube
and the cells were washed once in growth medium and then once in RPMI-1640. The
lymph node cells (2 × 10⁸) were fused with 6·75 × 10⁸ cells of the aminopterin-sensitive
heterohybridoma 53B3 (Tucker et al. 1984) using a standard fusion protocol
(Tucker et al. 1981). After fusion, the cells were resuspended in 133ml growth
medium and 1·0ml was distributed into each well of four 24-well plates (Costar,
USA). A control plate was also established which included wells containing
equivalent numbers of unfused lymph node cells and unfused 53B3. Plates were
incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 24h, 1 ml of
growth medium containing hypoxanthine (10mM), aminopterin (40mM) and
thymidine (1·6mM), was added to each well.
**Screening.** Hybridomas were screened for secretion of complement-fixing antibodies by the automated lymphocytotoxicity test (Kemp & Teale 1987). Briefly, PBM from the immunizing animal were stained with carboxy-fluorescein diacetate (Calbiochem, USA) and resuspended at a concentration of $3 \times 10^6$/ml. One microlitre of this suspension was then added to each well of a Terasaki plate (Greiner, West Germany) with 1 µl of culture supernatant. After 30 min at room temperature, 5 µl of rabbit complement (Cedar Lane, Canada) was added. After a further 60 min the reaction was stopped and counter-stained with propidium iodide (Sigma, USA) and the extracellular fluorescence quenched with ink (Ernst Leitz Wetzlar, West Germany). After a minimum of 20 min at 4°C the test was read on an automated fluorescence microscope.

**Cloning.** Cloning on soft agar was as described by Sachs et al. (1986).

**Ascitic fluid.** Antibody with a higher titre than that obtained from tissue culture supernatant was made from ascitic fluid prepared in Balb/c nude mice (Harlan-Olac, UK). Mice were inoculated intraperitoneally (i.p.) with pristane (2,6,10,14-tetramethyldecanoic acid (Aldrich, USA) 0·5 ml/mouse) and after 10 days were injected i.p. with $5 \times 10^6$ hybridoma cells. After a further 3–4 weeks, ascitic fluid was harvested.

**Antibodies.** The mAbs ILA4 (IgM) and ILA7 (IgG$_2a$) and the alloantiserum KKA004 were used in population and binding studies. The reactivities of these antibodies have been described elsewhere. ILA4 and KKA004 detect the KN104 specificity which, in Boran cattle, is closely associated with the BoLA specificity w10 (Kemp et al. 1988). ILA7 reacts with lymphocytes from all w10 animals and with many others (Bensaid et al. 1988). The murine IgG$_1$ mAb ILA2 detects bovine IgG (Naessens et al. 1988) and was used for immunoprecipitation.

**Binding inhibition.** The ability of one antibody to inhibit the binding of another was demonstrated by incubating target cells with an excess of one antibody, on ice and in the presence of 0·2% sodium azide, for 30 min and then adding the second antibody at an appropriate dilution. In the case of ILA70, it was then possible to detect its binding independently from that of a mouse mAb by means of the mAb ILA2. Binding of ILA2 was in turn detected with biotinylated anti-murine IgG$_1$ (Southern Biotechnologies, USA) followed by phycoerythrin-avidin (Becton-Dickinson, USA). In the case of ILA4, the mab was directly biotinylated by incubation with NHS-LLC-biotin (Pierce, Netherlands) (500 µg/mg) and its binding was detected by fluorescein-avidin (Vector, USA). Samples were analysed on a fluorescence activated cell sorter (FACSTAR PLUS, Becton-Dickinson). In both cases, appropriate controls were included to demonstrate the absence of cross-reactivity between the second step reagent and the mAb used for inhibition.

**Immunoprecipitation.** Since ILA70 does not bind to protein-A, antibody from ascitic fluid was adsorbed onto sepharose beads coupled with the anti-bovine IgG$_1$ mAb ILA2. That ILA70 successfully bound by this method was demonstrated by loading the sepharose/antibody complex onto an SDS-polyacrylamide gel. After running under reducing conditions the gel was stained with Coomassie blue. Two species of antibody were seen, the bovine antibody ILA70 having a heavy chain of higher molecular mass than ILA2 (Fig. 1).
Figure 1. Polyacrylamide gel electrophoresis of the bovine mAb ILA70 together with the murine mAb ILA2 (lane C) shows that the bovine heavy chain has a higher apparent molecular mass than its murine equivalent. The murine mAb ILA2 is shown alone and with normal mouse serum control (in lanes A and B) for comparison. Coomassie blue stain.

Immunoprecipitation was performed essentially as described by Bensaid et al. (1988). In brief, ILA70-ILA2-Sepharose was incubated with an NP40 lysate ('pre-lysis') of $^{125}$I surface-labelled PBM or *Theileria parva*-transformed lymphoblastoid cell lines (Brown et al. 1973), washed extensively and run in a 12.5% SDS-polyacrylamide gel under reducing conditions. After staining and drying, the gel was autoradiographed for 24 or 48h at $-70^\circ$C. Controls were provided by normal nude mouse serum and fetal bovine serum. Additionally, an initial immunoprecipitation was performed by the method of Naessens et al. (1985) which differs from that described above in that the cells are lysed after addition of antibody-sepharose ('post-lysis').

Results

**Generation of a bovine mAb**

Fifteen days post-fusion approximately 90% of the 96 wells contained one or more colonies of growing hybrids. Screening with PBM from the immunizing animal and with *T. parva*-transformed cell lines from two animals (one of which shared the w10 haplotype with the immunizing animal and one of which did not) showed a high level of antibodies specific for the w10 haplotype in all wells, including those containing unfused lymph node cells. After 3 weeks, during which time 50% of the medium in the wells was replaced by fresh medium every 2 days, the background levels dropped appreciably. At this time, approximately 20 cultures were selected as being potential
antibody secretors and were expanded into 25 cm² culture flasks. After another 3 weeks, antibody levels in the supernatant medium of all but one of these cultures had ceased to be detectable.

The secreting culture (designated BF2-3-2a) was cloned in soft agar. When the clones were screened, 30% were found to be antibody secretors. One of these clones was expanded and re-cloned and 80% of the subclones were found to be antibody secretors. One of these was expanded and re-cloned, and again 80% of the new subclones secreted antibody. One of these (BF2-3-2a.13.24.16) was designated ILA70. The cells from this clone were inoculated into nude mice; the resultant ascitic fluid typically gave 50% lysis of appropriate PBM in the cytotoxicity test at a dilution of 1:256. Tissue culture medium typically gave 50% lysis of appropriate PBM in the cytotoxicity test at a dilution of 1:32. The cloned cell line was expanded and cryopreserved. The cells continued to secrete antibody for several months at which time culture was discontinued.

Specificity of ILA70

Population data. ILA70 supernatant was used in the cytotoxicity test on PBM from 923 cattle of both B. taurus and B. indicus type. ILA70 reacted with PBM from 60 (15%) of these animals and its reactivity had a correlation coefficient of 0.7 with the cluster defined by the mAb ILA4 and the alloantiserum KKA004. These reagents detect KN104 — a specificity which is closely associated with w10 in B. indicus (Kemp et al. 1988). ILA70 showed no significant correlation with any other specificity except w10.

Binding inhibition. FACS analysis showed that all PBM from appropriate animals bound ILA70. ILA4 was shown not to inhibit the binding of ILA70 whereas ILA70 inhibited the binding of ILA4 (Fig. 2). The anti-w10 mAb ILA7 did not inhibit either ILA70 or ILA4 and binding of ILA7 was not inhibited by either ILA70 or ILA4.

Immunoprecipitation. Immunoprecipitation with PBM and T. parva-infected cell lines derived from the animal B641 which carries the w10-KN104 haplotype (Bensaid et al. 1998) showed that ILA70 detects a classical class I dimer of 44 and 12 kDa (Fig. 3). Sequential precipitations were performed to investigate the relationship between the molecule recognized by ILA70 and w10. Cell lysates were precleared and then exhaustively precipitated with ILA7. Subsequently, it was possible to precipitate class I molecules with ILA70 (Fig. 3). It was also shown that following clearance of antigen reactive with ILA70 to below detectable levels, class I molecules reactive with ILA7 were still present.

Discussion

The animal B641 which was used as a source of material for the immunoprecipitation and blocking studies expresses the locally defined A-locus specificity KN18 together with the w10 and KN104 specificities. It is known from studies of deletion mutants of B641 cell lines (Teale 1988) and from family and population studies (Bensaid et al.
Figure 2. FACS profiles showing that the binding of the bovine mAb ILA70 to a KN104-bearing cell-line is not inhibited by the murine mAb ILA4. In contrast, the binding of ILA4 to this cell line is strongly inhibited by the presence of ILA70.

Figure 3. Left gel: Class I antigen is precipitated by ILA70 from B641 lymphoblastoid cell line by two methods. Post-lysis in which cells are lysed after addition of antibody-sepharose and pre-lysis in which cells are lysed before addition of antibody-sepharose. The two techniques gave comparable yields. FBS (fetal bovine serum-sepharose) control does not precipitate labelled material. Right gel: Subsequent exhaustive precipitation from the pre-lysis lysate with ILA70 does not deplete the quantity of antigen which may then be precipitated by the anti-w10 mAb ILA7. There appear to be two antigencially distinct class I MHC products from a single haplotype.
1988; Kemp, unpublished observations) that ILA70, ILA7 and ILA4 do not react with products of the KN18 haplotype of the animal B641. Thus the sequential immunoprecipitations reveal two distinct class I products encoded by a single class I haplotype and the binding inhibition studies support the interpretation. This is entirely consistent with previous results (Bensaid et al. 1988) which also used cells derived from B641 and the mAb ILA7. However, the earlier study, which was carried out before the advent of ILA70, relied on an alloantiserum to precipitate the KN104 antigen.

The nature of the genetic control of these two antigens remains to be determined. The simplest explanation of the data presented here is that KN104 (the antigen detected by ILA70, ILA4 and ILA7) and w10 are encoded by separate class I loci on the same haplotype. This interpretation is supported by recent experiments in which murine L-cells were transfected with genomic DNA from a w10, KN104 homozygous animal and clones were isolated which express either w10 or KN104 but not both (Toye et al. 1990). However, it remains possible that these antigens represent alternative products of the same allele. It would certainly require a high linkage disequilibrium between two distinct loci to maintain the observed association between w10 and KN104 which is seen in diverse breeds of cattle (Kemp et al. 1988).

This is the first report of a bovine-anti-BoLA mAb and its success opens the way to the raising of more such reagents for use in studies of the bovine MHC. However, there are clearly serious problems to be overcome before the approach can become routine. The number of antibody secreting lines which arise from a fusion is very low. In subsequent fusions, using a similar protocol, many hybrids have grown but none have been found to secrete antibody. It is possible that improved immunization protocols will result in more primed B-cells suitable for fusion. Alternatively, means of specifically bringing together a fusion partner (reviewed in French et al. 1986) and a cell bearing antibody with specificity for the immunogen may prove fruitful.

We are not in a position to comment on whether or not instability of antibody-secreting bovine/murine hetero hybridomas will be a problem. Certainly in the case of ILA70 it was not, but it may be that many other antibody-secreting clones were lost in the first few days following the fusions. It would be expected that different fusion partners would have different characteristics and it may be useful to attempt to identify a partner which generates more stable hybrids.

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References


