

Quantitation of Bovine Immunoglobulin Isotypes and Allotypes using Monoclonal Antibodies

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ABSTRACT

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A panel of 10 monoclonal antibodies specific for bovine immunoglobulins M, A, G₁, G₂ and light chains were produced and enzyme-linked immunosorbent assays developed to measure Ig levels in body fluids and culture supernatants using this panel of MAbs. An inhibition ELISA was accurate and sensitive for MAbs of high affinity, detecting levels as low as 10 ng ml⁻¹ of IgM using a high-affinity MAb, IL-A50 (dissociation constant = $1.3 \times 10^{-11} M$). For MAbs of lower affinity (K_D of less than $0.25 \times 10^{-9} M$) a sandwich ELISA was more sensitive, detecting 0.1-1.0 µg ml⁻¹ Ig, provided a conjugate of an anti-light chain MAb was used.

Using these ELISA techniques, four pairs of MAbs specific for bovine IgM, IgA, IgG₁ and IgG₂ respectively, were screened on sera from over 100 cattle of different breeds to determine whether any detected a polymorphic epitope. MAbs IL-A30, IL-A60, IL-A66, IL-A71, IL-A72, IL-A73 and IL-A74 were shown to recognise monomorphic determinants on their respective heavy chains. In contrast, the epitope recognised on the µ-heavy chain by MAb IL-A50, which had previously been shown to be polymorphic, was found to be allelic and inherited under the control of a single gene, probably *Cu*.

INTRODUCTION

Two techniques are routinely used to measure total immunoglobulin (Ig) levels in cattle, the single radial immunodiffusion technique (SRID; Mancini, 1965) and the sandwich enzyme-linked immunosorbent assay (S-ELISA; Voller et al., 1975). The SRID test takes 3 days to develop and is a difficult test to operate accurately whereas the S-ELISA can be completed in a day and is both sensitive and accurate. Polyspecific antisera are usually used in these assays but to produce a good antiserum, highly purified antigen is required and the antiserum itself requires purification and frequently extensive absorption to

remove cross-reactivity. There is also considerable interbatch variation. Monoclonal antibodies (MAbs) on the other hand are highly specific, can be prepared in large quantities and there is little interbatch variation (Goding, 1980). Nevertheless because MAbs recognise single epitopes, they do not cross-link antigens sufficiently to form insoluble precipitates which are required in the SRID test. In the S-ELISA, the use of a MAb as both the catching antibody and the conjugate often results in incomplete binding of the conjugated second step MAb (Edwards, 1981; Van Zaane and IJzerman, 1984). Also some MAbs prove difficult to purify and enzyme-label satisfactorily (Jeanson et al., 1988). To overcome these problems we report an inhibition ELISA (I-ELISA) and a S-ELISA for quantifying bovine Ig levels using MAbs which we have produced. In addition we show that these techniques can be used to determine the frequency and distribution of polymorphic epitopes recognised by some MAbs.

MATERIALS AND METHODS

Animals

Bos indicus (Boran) and *Bos taurus* (N'Dama, Ayrshire/Guernsey crosses and Friesian) cattle were maintained indoors at ILRAD or kept under ranch conditions at the Institute's breeding farm at Kapiti Plains.

BALB/c and BALB/c × Swiss mice were bred and maintained in ILRAD's small animal unit, and used when they were 3 months old.

Ig purification

The IgG₁/IgA mixture used to immunise the mice was purified from colostrum by anion exchange using a DE52 (Whatman Biosystems, Maidstone, Kent) column according to the method of Fey et al. (1976). Pure Ig preparations (IgG₁, IgG₂, IgA and IgM) were made using immunoaffinity columns. MAbs were conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Serum or colostrum was run through each column and the bound fraction eluted using glycine/HCl 0.1 M, pH 3.0. To check the purity, each Ig was tested by the double immunodiffusion technique (Ouchterlony, 1958) using rabbit anti-bovine class specific antisera (Nordic Immunological Labs., Tilburg, The Netherlands), by ELISA and by SDS-PAGE using the technique of Maizel (1971).

Mabs

IL-A58, IL-A59, IL-A60, IL-A66, IL-A71, IL-A72, IL-A73 and IL-A74 were derived from BALB/c mice after immunisation with either an IgG₁/IgA mixture purified from colostrum or IgG₂ purified from serum. Mice were immunised with 100 µg of Ig in Freund's complete adjuvant. Twenty-eight days later these mice were killed and the spleen adoptively transferred into BALB/c mice which had been irradiated with 600 rads from a Cesium-137 source 24 h pre-

viously. Each recipient mouse was injected with 2×10^7 cells together with 25 μg Ig intravenously. Six days later the spleen cells from the recipient mice were fused with myeloma X63. Ag8.653 using established methods (Oi and Herzenberg, 1980). This technique was used to produce high affinity MAbs against the immunodominant determinants of each isotype (see Discussion). Hybridoma supernatants were screened by ELISA. Selected hybridomas were cloned and injected intraperitoneally into mice primed with pristane (Aldrich, Gillingham, Dorset, U.K.) to produce ascites fluid.

ELISA

The micro-ELISA technique first described by Voller et al. (1975) was used.

Sandwich ELISA. Polystyrene ELISA plates (Dynatech, Benkendorf, F.R.G.) were coated with 100 μl of MAb at a concentration of 10 $\mu\text{g ml}^{-1}$ in a carbonate/bicarbonate coating buffer 0.05 M, pH 9.6 and incubated at 37°C for 2 h. Plates were washed with PBS, pH 7.2, containing 0.05% Tween 20 (Sigma, Poole, Dorset, U.K.). The middle two rows of each plate were used for the serially-diluted standards. Each unknown sample was diluted in PBS/Tween, and 3 serial dilutions added to the plates, which were incubated for 2 h at 37°C. Plates were washed and alkaline-phosphatase (AP; Sigma) conjugated MAbs, diluted in PBS/Tween, added for a further 2 h at 37°C. The substrate p-nitrophenyl phosphate sodium (1 mg ml^{-1} ; Sigma) was added and the plates incubated for 30 min at 37°C. Optical densities were read using a Micro-ELISA reader (Model MR-580 Dynatech, Switzerland) at a wavelength of 405 nm.

Inhibition ELISA. To measure the Ig content of samples using the I-ELISA, serial dilutions of each sample were incubated with the MAb for 1 h at 37°C. 100 μl of this reactant was then transferred to a micro-ELISA plate, precoated with affinity purified Ig (0.1 $\mu\text{g well}^{-1}$). Plates were incubated for 2 h at 37°C, then washed in PBS/Tween and 100 μl of anti-mouse Ig conjugated to horseradish peroxidase (HRP; Amersham International, Amersham, U.K.), diluted to 1:2500, was added. After a further 2 h incubation at 37°C the plates were washed and 100 μl of 2,2' azino-bis (3-ethyl benz-thiazoline sulphonic acid; ABTS, Sigma) at 0.1 mg ml^{-1} in citrate buffer, pH 4 containing 0.0075% H_2O_2 , was added and plates were incubated for 30 min at 37°C. Optical densities were read at a wavelength of 405 nm.

Cross-inhibition ELISA (CI-ELISA). Plates were coated with 100 μl of 1 $\mu\text{g ml}^{-1}$ of Ig in coating buffer for 2 h at 37°C. After washing, the plates were incubated with 100 μl of a 1 $\mu\text{g ml}^{-1}$ solution of MAb for 2 h at 37°C. The plates were washed and then incubated with the alkaline-phosphatase conjugated MAbs for a further 2 h. The plates were then treated as described for the S-ELISA.

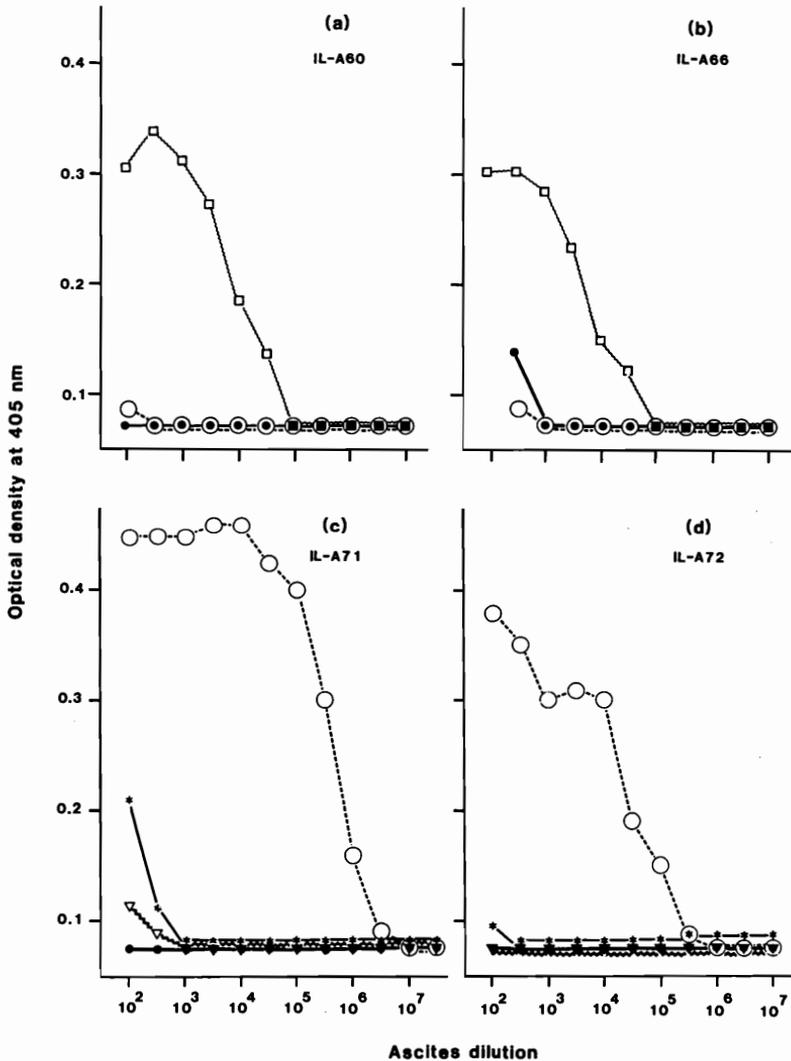


Fig. 1. Binding curves of MAbs IL-A60 (a), IL-A66 (b), IL-A71 (c), IL-A72 (d), IL-A73 (e), IL-A74 (f), IL-A58 (g) and IL-A59 (h) in ELISA, to plates coated with $1.0 \mu\text{g ml}^{-1}$ IgG₁ (□), IgG₂ (●), IgA (○), IgM (▽) or IgG (★).

Preparation of Ig standard for ELISA

Ig standards were made as described above. Each standard was adjusted to a protein concentration of 1 mg ml^{-1} in PBS and stored with 0.2% azide at 4°C . All were compared with a commercial standard (Miles Laboratories, Slough, U.K.).

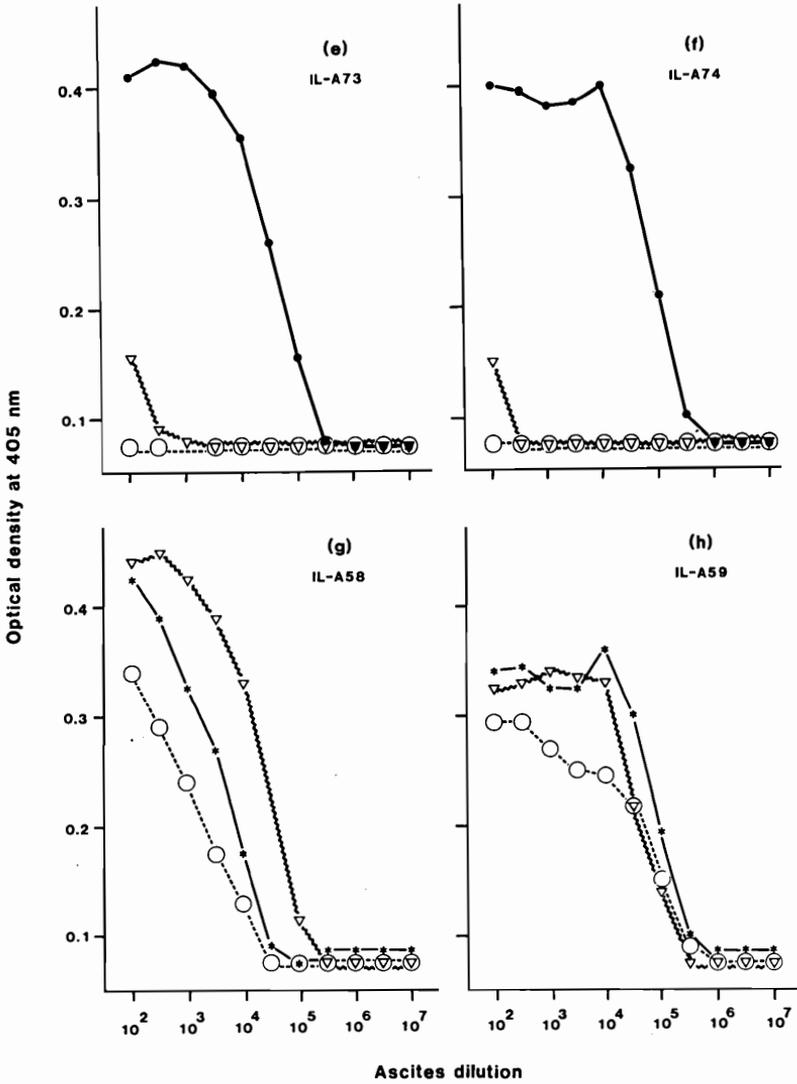


Fig. 1. Continued.

Alkaline phosphatase (AP) conjugation

Each MAb was conjugated to AP (Sigma) using the one-step glutaraldehyde method (Engvall and Perlmann 1971).

Calculations of dissociation constant (K_D)

The K_D was calculated using the modified I-ELISA, according to the method of Friguet et al. (1985).

TABLE 1

Summary of the specificity, murine isotype, dissociation constant, polymorphisms and detection range in the I-ELISA and S-ELISA for each MAb

Specificity:	Light chains		IgM		IgG ₁		IgG ₂		IgA	
MAb:	IL-A58	IL-59	IL-A30	IL-A50	IL-A60	IL-A66	IL-A73	IL-A74	IL-A71	IL-A72
Mouse isotype	IgG _{2a}	IgG ₁	IgG ₁	IgG _{2a}	IgG ₁	IgG ₁	IgG ₁	IgG ₁	IgG _{2a}	IgG ₁
Allotypes detected	—	—	—	+	—	—	—	—	—	—
K _D × 10 ⁹ M	1.4	1.9	0.25	0.013	1.2	1.2	11.5	18.5	0.041	0.27
Range in I-ELISA (μg ml ⁻¹)	ND	ND	0.25-1	0.01-0.1	1-15	1-15	10-50	10-250	0.1-0.6	0.5-2.5
Range in S-ELISA (μg ml ⁻¹)	ND	ND	0.1-1	0.1-1	0.1-5	0.1-1	0.1-1	0.1-1	0.1-5	0.1-5

K_D = dissociation constant calculated using the method of Friguet et al. (1985).

ND = not done.

FACS analysis

Analysis of cell surface immunoglobulin on peripheral blood lymphocytes was done by indirect immunofluorescence on a FACStar Plus (Becton Dickinson, Sunnyvale, CA) as described previously (Naessens et al., 1988).

Analysis of results

For the ELISA, standard curves were calculated for each plate from the mean of the titrated duplicate values. Unknown values were calculated using linear regression analysis. The chi-squared test was used to determine significance between observed and expected values in the family studies.

RESULTS

Characterisation of MAbs

Ten MAbs against bovine Ig isotypes were produced and characterised. The specificity of each MAb was confirmed by ELISA (Fig. 1) and by immunoadsorption using affinity-columns prepared with each MAb (Fig. 2).

In the ELISA, MAbs IL-A60 and IL-A66 bound specifically to IgG₁ (Fig. 1a and 1b), IL-A71 and IL-A72 to IgA (Fig. 1c and 1d) and IL-A73 and IL-A74 to IgG₂ (Fig. 1e and f). Two MAbs, IL-A58 and IL-A59 which bound to all bovine Ig isotypes (Fig. 1g and 1h) were also identified. MAbs specific for IgM, IL-A30 and IL-A50, and total IgG, IL-A2, were described previously (Naessens et al., 1988).

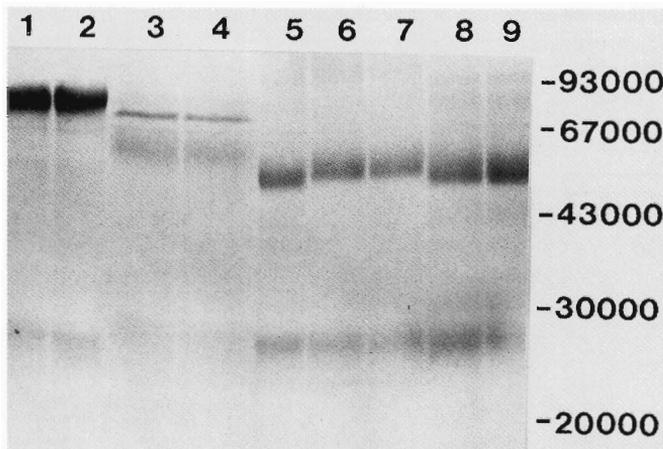


Fig. 2. SDS-PAGE of serum Ig bound by affinity columns of MAbs against IgM: IL-A30 (lane 1), IL-A50 (lane 2); against IgA: IL-A71 (lane 3), IL-A72 (lane 4); against IgG: IL-A2, (lane 5); against IgG₁: IL-A60 (lane 6), IL-A66 (lane 7); against IgG₂: IL-A73 (lane 8) or IL-A74 (lane 9).

Analysis by SDS-PAGE (under reducing conditions) of proteins eluted from the immuno-affinity columns (Fig. 2) revealed that IL-A60 and IL-A66 precipitated a major protein band of 55–59 000 Mw and that IL-A73 and IL-A74 recognised a 52–59 000 Mw protein, consistent with the heavy chains of IgG₁ and IgG₂ respectively. IL-A71 and IL-A72 each precipitated one band at 61–63 000 Mw and another at 75 000 Mw, consistent with the heavy chain and the secretory component of IgA. In addition, all six MAbs precipitated the Ig light chain of 22–25 000 Mw.

The dissociation constant (K_D) and mouse Ig isotype were determined for each MAb and are shown in Table 1.

Cross-inhibition

Cross-inhibition ELISAs, in which unconjugated MAbs competed with conjugated MAbs for binding to the antigen, were used to determine whether each pair of isotype-specific MAbs recognised the same epitope or epitopes situated in close proximity on the Ig molecule. The results are shown in Table 2. IL-A30 inhibited the binding of IL-A50 to IgM and vice versa. IL-A59 which recognises bovine light chains could also prevent both anti-IgM MAbs (IL-A30 and IL-A50) from binding to their respective antigens. The opposite was not true, IL-A30 and IL-A50 did not inhibit binding of IL-A59 to bovine IgM.

MAbs IL-A60 and IL-A66, which react with IgG₁, partially blocked the binding of each other. The two MAbs against IgG₂, IL-A73 and IL-A74, completely

TABLE 2

Cross-inhibition ELISA showing presence or absence of interference with binding between MAbs recognising the same isotype

Unlabelled MAbs	Conjugated MAbs									
	IL-A30	IL-A50	IL-A58	IL-A59	IL-A60	IL-A66	IL-A71	IL-A72	IL-A73	IL-A74
IL-A30	+	+	0	0	–	–	–	–	–	–
IL-A50	+ / 0	+ / 0	0	0	–	–	–	–	–	–
IL-A58	0	0	+ / 0	0	0	0	0	0	0	0
IL-A59	+	+	0	+	0	0	0	0	0	0
IL-A60	–	–	0	0	+	+ / 0	–	–	–	–
IL-A66	–	–	0	0	+ / 0	+	–	–	–	–
IL-A71	–	–	0	0	–	–	+	0	–	–
IL-A72	–	–	0	0	–	–	0	+	–	–
IL-A73	–	–	0	0	–	–	–	–	+	+
IL-A74	–	–	0	0	–	–	–	–	+	+

+, complete inhibition;

0, no inhibition;

+ / 0, partial inhibition;

–, not done.

inhibited each other. In contrast MAbs IL-A71 and IL-A72, specific for IgA, showed no cross-inhibition and therefore must recognise two different epitopes. Both MAbs recognised serum IgA and thus were recognising a determinant on the heavy chain not on the secretory component. MAbs IL-A58 and IL-A59 did not interfere with the anti- γ or anti- α MAbs.

Absorptions using immunoaffinity columns

A pool of serum from 10 animals was run through the IL-A58 immunoaffinity column until no more protein bound. When the residual serum fraction was then run through either the IL-A59, IL-A30 or IL-A2 columns, no more Ig bound. Likewise when serum was run exhaustively through the IL-A59 column, no more Ig was detected by the IL-A58, ILA30 or IL-A2 columns. Therefore IL-A58 and IL-A59 appeared to recognise epitopes common to lambda and kappa light chains.

No Ig from serum which had been exhaustively run through the IL-A73 (anti-IgG₂) column bound to the IL-A74 column or vice versa. When IgG, which had been purified from a pool of serum using IL-A2, was then run through the IL-A60 or IL-A66 columns to remove the IgG₁, all the remaining IgG bound to either the IL-A73 or IL-A74 columns.

Quantitative assays

The I-ELISA was developed to calculate levels of Ig in sera and in culture supernatants from in vitro lectin-activated B-cells. A standard curve was calculated for each MAb (Fig. 3) from which unknown values could be read. Each

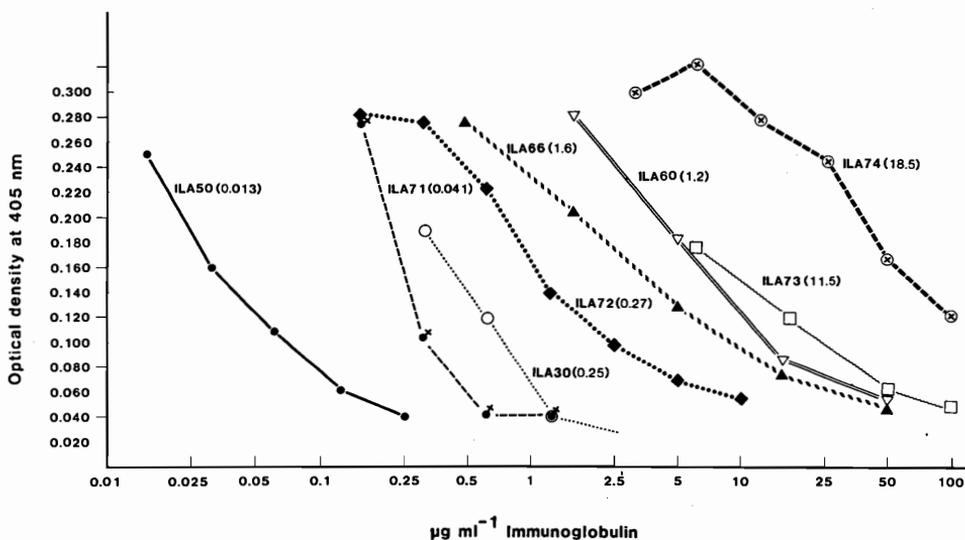


Fig. 3. Standard inhibition curves for each MAb in the ELISA. $K_D \times 10^9 M$ is shown in brackets.

standard curve was characteristic of, and repeatable for, each MAb. The slope of the curve and sensitivity of detection appeared to be determined by the dissociation constant of each MAb. The higher the affinity of a MAb, the steeper the slope of its standard curve, and the lower levels of Ig which could be detected. Thus IL-A50 had the highest affinity constant of 1.3×10^{-11} M and detected between 10 and 100 ng ml⁻¹ IgM. In contrast IL-A74 had the lowest affinity of 1.85×10^{-8} M and detected between 10 and 250 µg ml⁻¹ of IgG₂.

Although this assay was extremely sensitive with high affinity MAbs, it was insensitive and less accurate with MAbs of weaker affinity. Therefore a S-ELISA was developed as an alternative for use with low affinity MAbs. However the cross-inhibition assays described above revealed that many of the MAbs either partially or completely blocked the binding of each other. In the classic S-ELISA this resulted in the incomplete binding of conjugated MAb to bound bovine Ig (results not shown). Therefore either the IL-A58 or IL-A59 AP conjugates were used as the second step MAbs in all S-ELISA, since the binding of these MAbs was not inhibited by any other MAb. The detectable range of Ig in the S-ELISA is shown for each MAb in Table 1.

Calculation of Ig levels in commercial standards using the I-ELISA and the S-ELISA

Ig levels in commercially prepared standards, supplied for use in SRID kits, were calculated using S-ELISA and I-ELISA, to determine the accuracy of the ELISA techniques and to verify our internal standards. The results are shown in Table 3. The levels calculated by both assays and using each MAb were close to the concentrations given by the manufacturers except in assays using MAb IL-A50. IL-A50 detected a significantly lower level of IgM than IL-A30 and this level was also lower than that quoted by the supplier. This suggests that

TABLE 3

Comparison of Ig levels in a commercial standard using either the sandwich ELISA or inhibition ELISA

MAb	I-ELISA (SD) (mg/ml)	S-ELISA (SD) (mg/ml)	Manufacturer's stated level (mg/ml)
IL-A30	0.94 (0.02)	0.95 (0.06)	1.0
IL-A50	0.097 (0.02)	0.084 (0.034)	1.0
IL-A60	3.8 (0.23)	2.1 (0.3)	3.0
IL-A66	2.2 (0.2)	3.4 (0.2)	3.0
IL-A71	0.34 (0.05)	0.29 (0.03)	0.25
IL-A72	0.26 (0.08)	0.28 (0.03)	0.25
IL-A73	0.22 (0.08)	0.26 (0.04)	0.25
IL-A74	0.25 (0.08)	0.38 (0.03)	0.25

TABLE 4

Phenotypes of offspring from heterozygous parents

Phenotypes ^a of parents		Number of offspring			χ^2	Level of significance
		+ / +	+ / -	- / -		
+ / + × + / -	Observed	12	15	0	0.33	0.56
	Expected ^b	13.5	13.5	0		
+ / - × + / -	Observed	2	6	2	0.4	0.82
	Expected	2.5	5	2.5		

^aPhenotypes: + / +, animals homozygous for the IL-A50 allotype; + / -, heterozygous animals; - / -, animals lacking IL-A50 in both haplotypes.

^bExpected numbers for Mendelian inheritance of the marker.

which were *B. indicus* and 10% *B. taurus* cattle. IL-A50 alone was shown to recognise a polymorphic epitope, both by FACS analysis (Naessens et al., 1988) and by the S-ELISA for serum IgM. To determine if the epitope recognised by IL-A50 was an allelic determinant, genetics studies were carried out. The ILRAD breeding herds were screened and two bulls were identified as being heterozygous for the IL-A50 determinant, Bull 51 (*B. indicus*) and ND7 (*B. taurus*). Thirty-seven offspring of these bulls and their dams were tested to determine their phenotypes. The results obtained both by FACS and ELISA analysis are shown in Fig. 4. When IL-A30 and IL-A50 detected equal numbers of peripheral blood B-cells and similar serum IgM levels, then an animal was considered to be homozygous. When IL-A50 detected at least 30% fewer B-cells than IL-A30 and less than 50% of serum IgM, animals were classed as heterozygous. Two animals, in which IL-A50 recognised less than 2% of their circulating B-cells and detected no serum IgM, were considered to be homozygous negatives for the polymorphic epitope.

The phenotypes of the 37 calves are shown in Table 4, together with the expected distribution for Mendelian-type inheritance. The distribution of phenotypes obtained from our analysis is not significantly different to that predicted for Mendelian inheritance.

DISCUSSION

Four pairs of MAbs were selected for their specificity for bovine IgG₁, IgG₂, IgA and IgM by ELISA and by affinity chromatography techniques (Figs. 1 and 2). Two MAbs, IL-A58 and IL-A59, which bound to all Ig isotypes in ELISA and reacted with the light chain band in a Western blot (results not shown), were also included in the panel.

Using a competition assay the affinity constants of each MAb were calculated (Table 1). They ranged from weak (K_D of $18.5 \times 10^{-9} M$) to very strong (K_D of $0.013 \times 10^{-9} M$). To produce high affinity MAbs, we used a low immunising dose of Ig, since this is known to select for high affinity antibodies (Roitt et al., 1985). However this can result in the growth of small numbers of hybridomas. To overcome this problem, other authors have used several antigen boosts to increase the yield of hybridomas (Stahli et al., 1980; Van Zaane and IJzerman, 1984). However we have found that following repeated doses of antigen, although fusions yielded high numbers of hybridomas, their antibodies generally reacted with minor determinants (Naessens and Newson, unpublished results). Therefore we employed the technique of adoptive transfer of primed spleen cells into an irradiated host together with an antigen boost. This resulted in the production of MAbs of high affinity against immunodominant epitopes and a high frequency of producer clones comparable to that described by Stahli et al. (1980).

The CI-ELISA was used to obtain more information about which sub-class specific determinants, recognised by each pair of MAbs, were identical or located in adjacent positions. The two MAbs against bovine γ_2 (IL-A73, IL-A74) completely blocked the binding of one another and might therefore detect the same epitope. The two MAbs against bovine γ_1 (IL-A60, IL-A66) only partially inhibited each other, even when an excess of MAb was used to compete. Since IL-A60 and IL-A66 could completely inhibit their own binding, and since their affinity constants were very similar, we concluded that the two MAbs probably recognise adjacent but different epitopes, and that the partial inhibition is caused by steric hindrance. Also the two MAbs against bovine IgM recognise different epitopes, since IL-A30 detected a monomorphic and IL-A50 a polymorphic determinant (Naessens et al., 1988), but in close proximity, since the two MAbs inhibited each other. The two MAbs against bovine α -chains (IL-A71, IL-A72) did not inhibit one another, and therefore detect different isotypic determinants. The same was true for the anti-light chain MAbs, IL-A58 and IL-A59, and therefore at least two different isotypic epitopes coexist on all bovine light chains.

Although heavy chain isotypes are usually located on the Fc fragment, in one case an anti-light chain MAb interfered with binding of two MAbs to a heavy chain isotype: MAb IL-A59 could prevent binding of IL-A30 and IL-A50 to bovine IgM, but the opposite was not true, i.e., IL-A30 and IL-A50 did not prevent the anti-light chain from binding to IgM. This suggests that either the light chain and μ -chain epitopes are situated close together, or that binding of IL-A59 to the light-chain alters the configuration of the Ig molecule in such a way that the μ -chain epitopes recognized by IL-A30 and IL-A50 are masked. Van Zaane and Hulst (1987) found a similar phenomenon using MAbs generated against porcine Igs.

The CI-ELISA enabled us to predict that the isotype specific AP-conjugated

MAbs would not be suitable for use in the S-ELISA. Polyspecific anti-sera contain a large number of antibodies of different epitope specificities which can bind alternative epitopes to those blocked by capturing antibody (Goding, 1980). Since MAbs recognise single epitopes, they generally block the binding of the homologous second step MAb (Edwards, 1981). Therefore, we used the anti-light chain MAbs as our conjugate step in the S-ELISA to overcome this problem. Both the S-ELISA and the I-ELISA here gave accurate, reliable results which correlated with an independent standard (Table 3). The choice of assay depended on the degree of sensitivity required, which, in the I-ELISA, was determined by the affinity of the MAb. MAbs specific for bovine Igs have been reported by several other groups (Pinder et al., 1980; Van Zaane and IJzerman, 1984; Letesson et al., 1985; Naessens et al., 1988; Thatcher and Gershwin, 1988). The S-ELISA and I-ELISA can be used with these MAbs to quantitate bovine Igs and detect whether any of these MAbs recognise polymorphic epitopes.

Our panel of MAbs was tested on sera from over 100 cattle of different breeds to find possible polymorphisms. No polymorphic determinants were detected, other than by IL-A50 (Naessens et al., 1988). Allotypic determinants have been described for bovine Ig isotypes: the C1 allotype on the IgG₁ heavy chain was used to study the transfer of maternal Igs to offspring (Rapacz and Hasler-Rapacz, 1972; Wegrzyn, 1973; Wegrzyn and Wegrzyn, 1978). The A1/A2 allotypes on the Fc portion of IgG₂ (Blakeslee et al., 1971a,b) and allotypes on bovine light chains (Blakeslee et al., 1971a,b; Faber and Stone, 1976) have also been reported. The anti-IgG₁ MAbs (IL-A60 and IL-A66) and the anti-IgA MAbs (IL-A71 and IL-A72) each recognised a different epitope to its partner. Therefore if either MAb recognised a polymorphic epitope it would have become apparent during the screening of large numbers of cattle, since one MAb would recognise a lower level of Ig compared to its partner unless, in the unlikely circumstance, both MAbs detect different allotypes which always occur together. No such discrepancies have so far occurred. However, the possibility cannot be ruled out that any one of the panel of MAbs may recognise a polymorphic epitope whose negative allele was not encountered in the animals tested.

Since IL-A73 and IL-A74 might recognise the same epitope on the IgG₂ heavy chain, we cannot rule out the possibility that they detect a polymorphic determinant. The IgG₂ heavy chain is known to express two alleles, A1 and A2, with the A1 allele expressed in 80% of cattle (Butler and Heyermann, 1986). Moreover two sub-classes of IgG₂ have recently been described (Butler et al., 1987). It is possible that IL-A73 and IL-A74 recognise only one of these subclasses or one allotype. However, several pieces of evidence argue against the possibility that these MAbs recognise only a proportion of bovine IgG₂. Firstly, both MAbs detected the same amount of IgG₂ in the commercial standard and these levels correlated with the manufacturer's, in contrast to those of IL-A50. Secondly,

when a pool of serum was run through either the IL-A73 or IL-A74 immunoaffinity columns until no more bound and was then tested with a polyspecific anti-IgG₂ sera, no more IgG₂ was detectable (results not shown). Finally, the cross-absorption studies using affinity chromatography showed that all the bovine IgG₂ in a pool of serum bound to the IL-A73 and IL-A74 immunoaffinity columns. Thus IL-A73 and IL-A74 appear to recognise an epitope which is common to all bovine IgG₂.

It has previously been reported that IL-A50 detects a polymorphic epitope on cell surface IgM (Naessens et al., 1988). The results described in this paper indicate that this polymorphic epitope is also present on serum IgM (Fig. 4). This means that IL-A50 cannot be used to screen IgM levels in large numbers of cattle. However, as it is a MAb of very high affinity, it is extremely useful for detecting low levels of IgM secreted in culture from lectin-activated or specific antigen-stimulated B-cells. But each animal must be tested prior to such assays and shown to be homozygous positive for the IL-A50 epitope, by comparing the IgM concentration measured with IL-A50 with that measured by IL-A30.

B5/4, a widely available anti-IgM MAb (Pinder et al., 1980), is also known to recognise a polymorphic determinant on IgM (Naessens et al., 1988). This probably accounts for the discrepancies between levels of B-cells positive for IgM detected by B5/4 and the levels detected by a polyclonal antiserum described by Fossum et al. (1988). In the genetic studies, B5/4 was inherited in the same way as IL-A50 (data not shown) and therefore also detects an IgM allotype.

The family studies (Fig. 4) revealed that, in general, there was a good correlation between the number of B-cells recognised by IL-A50 and the levels of IL-A50 positive serum IgM compared to levels detected by IL-A30. However, in some animals (Fig. 4, points marked with an asterisk) although IL-A50 detected a lower percentage of B-cells than IL-A30, it recognised the same amount of serum IgM as IL-A30. It is possible that in these cattle IL-A50 positive B-cells secrete more IgM than B-cells expressing the IL-A50 negative allele. In view of this, we considered that the expression of the polymorphic epitope on cell surface IgM was a more accurate assessment of whether an animal was heterozygous or homozygous for the IL-A50 epitope.

Analysis of the phenotypes of offspring from the two heterozygous bulls, and either homozygous or heterozygous dams (Table 4), revealed that the epitope recognized by IL-A50 was an allotype which was defined by one gene. The gene for this allotype is not linked to the bovine MHC complex (BoLA) and is probably the *Cu*-gene. The fact that the two alleles were expressed in both *B. taurus* and *B. indicus* cattle suggests that a mutation occurred early in the history of the bovid group and has been conserved.

The two quantitative ELISA techniques in conjunction with the panel of MAbs, described in this paper, will be useful in measuring bovine Ig levels in

body fluids and supernatants from in vitro cell culture. The anti-allotype antibodies, IL-A50 and B5/4, can only be used on genetically or serologically characterised animals, but they may be useful as tools to study the genetics and expression of Ig genes.

Hybridoma cells from the monoclonal antibodies described here are being sent to the American Type Culture Collections and the European Collection of Animal Cell Cultures.

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