Monoclonal Antibodies against Bovine Immunoglobulins and their Use in Isotype-Specific ELISAs for Rotavirus Antibody

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Monoclonal antibodies (MCA) against bovine immunoglobulin (BIg) isotypes were produced and characterized. MCAs were obtained which react specifically with IgG, IgG_1, IgG_2 or IgA while MCAs against IgM showed a partial cross-reaction with affinity purified IgA.

MCAs with optimal characteristics for application in ELISA were selected and used as conjugates in an indirect double antibody sandwich assay (IDAS) and as the capturing antibody in an antibody capture assay (ACA) for the isotype-specific detection of antibodies against rotavirus.

Based on theoretical grounds, experimental analysis of inter- and intra-isotype competition in IDAS and ACA, respectively, and a direct comparison of both tests, the IDAS was selected for the detection of IgG_1 and IgG_2 anti-rotavirus antibodies. The ACA was the test of choice for the detection of IgM and IgA anti-rotavirus antibodies. The isotype specificity of these tests relies on the specificity of the MCAs and was confirmed for each test by the observation that samples containing rotavirus antibodies of only 1 particular isotype reacted only in the homologous assay. The MCAs against bovine Ig isotypes and isotype-specific ELISAs were found to be very useful in the study of humoral mucosal immunity in calves infected with rotavirus.

Introduction

Antibodies bathing mucosal surfaces play an essential role in protecting animals and man against invading pathogens from their environment. For the intestinal mucosa, these antibodies may be passively acquired through colostrum and milk or locally produced by elements of the mucosal immune system. Also, transudation of serum immunoglobulins can occur. In most species, immunoglobulin A (IgA) is the major immunoglobulin in milk and mucosal secretions (Tomasi and Zigelbaum, 1963; Heremans, 1974). However, in ruminants, the predominating role of IgA in secretions is under question since IgG_1 seems to be the most abundant immuno-

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globulin in milk and intestinal secretions. Furthermore it appears to be (partly) serum derived (Newby and Bourne, 1976; Morgan et al., 1981; Newby et al., 1982).

To study the mucosal immune system of ruminants and especially the intestinal defence against enteric infections such as rotavirus induced enteritis, isotype *-specific ELISAs for the detection of antibodies against a specific pathogen are very useful. They are sensitive and capable of handling large numbers of samples. In addition, the samples need not to be separated into their constituent Ig isotypes. Obviously, the reliability of these tests depends largely upon their isotype specificity, which has to be demonstrated for each reagent and each test. This means that isotype specificity should be checked in the test under study or a similar test with the same sensitivity and that it is not sufficient to check specificity of antisera in less sensitive tests, such as immunodiffusion or immunoelectrophoresis. For instance, in earlier experiments with conventional antisera against bovine Ig (BIg) isotypes, we observed marked cross-reactions in ELISAs although the antisera appeared to be monospecific in immunodiffusion tests (see also Sloan and Butler, 1978; Townsend et al., 1982). An inherent problem in isotype-specific ELISAs is the competition that occurs. This may be between isotypes (inter-isotype competition), when anti-Ig isotype-specific reagents are used as conjugate, or within an isotype (intra-isotype competition) when anti-Ig isotype-specific reagents are used as the capturing anti-bodies (Chantler and Diment, 1981). Thus, in the former case a (weak) IgA or IgM response may be missed due to the presence of competing IgG. In the latter case samples containing equal amounts of specific antibody of a particular isotype may yield different results in ELISAs if the ratio of the specific antibody to the total amount of this isotype differs in these samples. The extent of inter-isotype competition appears to be influenced by antibody concentration and affinity and to vary with the antigen under study (Filice et al., 1980; Townsend et al., 1982).

To ensure the specificity and availability of isotype-specific reagents we produced and characterized monoclonal antibodies against the bovine Ig isotypes. With these reagents as conjugated or capturing antibodies an indirect antibody sandwich assay (IDAS) and an antibody capture assay (ACA) were developed for the isotype-specific detection of bovine antibodies against rotavirus. The optimal test for each isotype was selected on the basis of inter- and intra-isotype competition analysis and a direct comparison of the IDAS and ACA. Finally, evidence for the isotype specificity of each test is presented.

Materials and Methods

Materials

Polyethylene glycol 4000 (PEG) (for gas chromatography), dimethylsulfoxide (DMSO) p.a., and Tween 80 were obtained from Merck, Meppel.

Pristane (2,6,10,14-tetramethylpentadecane) was obtained from Janssen Chimica, Beerse.

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* Where appropriate the word 'isotype' includes subclasses IgG_1 and IgG_2.

Animals

BALB/c mice (SPF) were obtained from CPB-TNO, Zeist, and were bred for up to 2 generations in our Institute.

Bovine immunoglobulins

Bovine immunoglobulin preparations were gifts from Dr. B.A. Bokhout, Central Veterinary Institute, Immunology Department, Lelystad (IgG_1, IgG_2, IgM and IgA) and from Ms. A. Noordzij, State University of Utrecht, Faculty of Veterinary Science, Utrecht (IgG_1, IgG_2 and IgM). IgG_2 was also obtained from Miles Laboratories, Elkhart, IN. Results from immunodiffusion tests performed by the suppliers of the Ig preparations indicated that each preparation was pure under their test conditions.

Antisera

Rabbit or goat antisera against mouse Ig isotypes G_1, G_{2a}, G_{2b}, G_3, M and A were obtained from Nordic Immunological Laboratories, Tilburg. Rabbit antisera against bovine IgG, IgM and IgA were obtained from Miles Laboratories, Elkhart, IN. For the preparation of swine anti-rotavirus serum an orally infected SPF piglet was hyperimmunized intramuscularly with purified porcine rotavirus.

Viral antigen

The rotavirus preparation used in ELISA consisted of a pool of fecal samples obtained after oral infection of an SPF calf with rotavirus. Specific ELISAs for the detection of rotavirus (Ellens and De Leeuw, 1977), coronavirus (Ellens et al., 1978) and the K99 antigen of *E. coli* (Ellens et al., 1979) indicated that the preparation contained 10^3 ELISA units of rotavirus and was free of bovine coronavirus and the K99 antigen of *E. coli*.

A pool of fecal samples free of the above mentioned agents was used as a control antigen preparation.

Production of monoclonal antibodies

For each bovine Ig isotype BALB/c mice of at least 2 months of age were hyperimmunized by intraperitoneal (i.p.) injection of 10–100 μg of BIg preparation adsorbed onto aluminium hydroxide. Two to 8 weeks after the last injection 1 of the mice was repeatedly injected (i.p. and intravenously) with daily doses of 200–400 μg of BIg according to the method of Stähli et al. (1980). On the day following the last injection spleen cells were collected and fused with P3 X 63-Ag8.653 myeloma cells (Kearney et al., 1979). The fusion protocol of Fazekas de St.Groth and Scheidegger (1980) was followed with minor modifications. Myeloma cells were grown in suspension culture with RPMI 1640 based medium containing 15–20% fetal calf serum. Thus, cells in the logarithmic growth phase were obtained which could easily be harvested. Spleen cells and myeloma cells were washed in calcium-free medium (Eagle's essential medium for suspension culture with 0.01% w/v EDTA; Ca^{2+} increases the toxicity of PEG, Schneiderman et al., 1979). The fusion solution was also made up with this medium and contained 50% w/v PEG 4000 and 5% w/v

DMSO. After fusion the cells were washed once with calcium-free medium, suspended in HAT medium and seeded in microtissue culture plates (Costar no. 3596). Positive cultures were subcloned once or twice by limiting dilution, until supernatants of at least 90% of the wells with growing cells were positive in the screening test. Subclones were always picked from wells which contained only 1 visible colony at the first microscopic inspection of the cultures. Mouse peritoneal cells were used throughout as feeder cells. Ascites fluids containing MCAs were obtained from pristane treated BALB/c mice, injected 5–21 days before with $1-2 \times 10^6$ hybridoma cells. The first figure of the MCA code refers to the fusion number.

ELISA (see Fig. 1)

General. Polystyrene ELISA plates (Dynatech M129A) were coated with antigen or antibody in 50 mM $NaHCO_3$, pH 9.6 for 16 h at 37°C. All other reagents except the substrate were diluted in ELISA buffer (0.5 M NaCl, 10 mM PO_4^{3-}, pH 7.2. 0.05% w/v Tween 80) and were added at predetermined optimal concentrations in a volume of 100 μl. Conjugate solutions contained 4% horse serum to reduce the background. Following each incubation step, plates were washed 10 times with 0.05% w/v Tween 80 in demineralized water using a home-made ELISA plate washing device. As the final step in all ELISAs recrystallized 5-aminosalicylic acid (Ellens and Gielkens, 1980) was used as substrate. Three to 16 h after addition of substrate absorbance was measured in a Titertek Multiscan at 450 nm.

Indirect ELISA. For the screening and characterization of MCAs against bovine Ig isotypes an indirect ELISA was used. ELISA plates were coated with BIg preparation (1–10 μg/ml). Culture medium or ascites fluid diluted at least 1 : 1 in ELISA buffer was added and incubated for 1 h at 37°C. Subsequently rabbit anti-mouse Ig conjugated with horse-radish peroxidase (HRP) (Nordic, Immunological Laboratories, Tilburg) was added and incubated for 1 h at 37°C.

DAS-ELISA. For the detection and quantitation of BIgs, a double antibody sandwich (DAS) assay was used. Plates were coated with monoclonal antibody
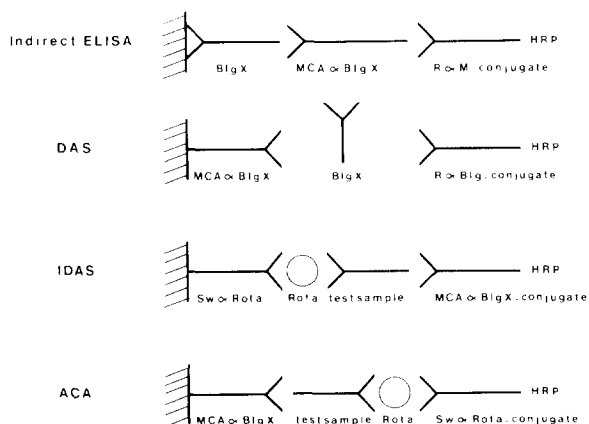

Fig. 1. Schematic diagram of ELISA tests used. X stands for isotype G, G_1, G_2, M or A.

purified from ascites fluid by ammonium sulfate precipitation. Two-fold dilutions of BIg isotype standards and test samples were added and incubated for 2 h at 37°C. A mixture of rabbit antisera against bovine IgG, IgM and IgA conjugated with HRP using a modification of the method of Wilson and Nakane (1978). The concentration of BIg in test samples can be calculated from titration curves.

Isotype-specific ELISAs for the detection of rotavirus antibody. An indirect double antibody sandwich assay (IDAS) and an antibody capture assay (ACA) using monoclonal bovine Ig isotype-specific reagents were developed for the detection and titration of Ig isotypes with antibody activity against rotavirus in serum, colostrum, milk or feces. For the IDAS, plates were coated with swine anti-rotavirus immunoglobulins. To horizontal rows B to H rotavirus was added and to row A a control fecal sample was added. Following incubation for 1.5 h at 37°C, 2-fold dilutions (starting at 1/20) of test samples in ELISA buffer containing 10% horse serum were added to wells B–H of vertical rows. Well A was filled with a 1/20 dilution of the test sample (control without rotavirus). In the case of fecal test samples feces was mixed with 4 vols. of ELISA buffer and the suspension was centrifuged for 10 min at $250 \times g$ to remove solid material. The supernatants were regarded as undiluted test samples. Plates were incubated for 2 h at 4°C. Subsequently, HRP-conjugated MCA specific for the BIg isotype under study was added and incubated for 1 h at 37°C. For the ACA, plates were coated with MCA specific for the BIg isotype under study. MCAs were purified by ammonium sulfate precipitation from ascites fluid. To wells B–H of each vertical row 2-fold dilutions (starting at 1/20) of test samples in ELISA buffer were added. Well A was filled with a 1/20 dilution of the test sample (control without rotavirus, see below). After incubation for 2 h at 37°C, rotavirus was added to rows B–H and a control fecal sample was added to row A. Plates were incubated for 1.5 h at 37°C and subsequently swine anti-rotavirus antibodies conjugated with HRP were used as conjugate. Incubation was for 1 h at 37°C.

Affinity chromatography

Five to 10 mg of MCA purified by ammonium sulfate precipitation from ascites fluid were coupled to 1.0 ml of cyanogen bromide (CNBr)-Sepharose 4B (Pharmacia) activated according to the suppliers manual. Columns (0.5–1.0 ml) were pre-run with 5 ml starting buffer (0.1% horse serum in phosphate-buffered saline containing 0.05% w/v Tween 80) and with 5 ml of 2 M glycine-HCl, pH 2.1. After re-equilibration with starting buffer, a preparation of BIg isotype in starting buffer was loaded onto the column and the unbound fraction was collected. Bound BIg was eluted with 2 M glycine HCl, pH 2.1 and the eluate was immediately neutralized with 1 vol. of 3 M Tris, pH 8.0.

Results

Monoclonal antibodies against bovine Ig-isotypes

Hybrid cells producing monoclonal antibodies against bovine Ig isotypes were

obtained after fusion of myeloma cells with spleen lymphocytes from mice immunized with purified $BIgG_1$ (fusion 15), $BIgG_2$ (fusions 11 and 12), BIgM (fusion 17) or BIgA (fusion 16). After subcloning once or twice, stable subclones were grown as ascites tumors in BALB/c mice. MCAs in ascites fluid were characterized by the indirect ELISA for their reactivity with BIg isotypes and by immunodiffusion tests for their mouse isotype (Table I and Fig. 2). The results of the ELISAs showed that several MCAs reacted monospecifically with IgG, IgG_1, IgG_2 and IgA, while other

TABLE I

CHARACTERIZATION OF MCAs AGAINST BIg ISOTYPES

Specificity	Clone code	Mouse isotype	Titer against [a]				Detection [b] limit
			G_1	G_2	M	A	
BIgG	11.4	G_1	(4) [c]	6	--	–	NT [d]
	11.5	G_1	(5) [c]	6	--	–	NT
	11.6	G_1	(5) [c]	6	...	–	NT
	12.2	G_1	5	5	--	–	NT
	12.4	G_1	5	6	–	..	NT
	12.6	G_1	5	6	–	...	NT
	12.11	G_1	5	6	–	–	NT
$BIgG_1$	15.8	G_{2a}	5	–	...	–	10
	15.17	G_{2a}	4	–	–	–	30
	15.18	M	4	2	-	...	>1000
$BIgG_2$	12.1	G_1	--	6	–	–	>1000
	12.5	G_1	--	6	.	--	10
BIgM	17.3	A	2	2	6	3	30
	17.4	A	2	2	6	2	30
	17.11	G_1	–	–	6	2	30
BIgA [e]	16.4	G_1	–	–	–	5	20
	16.24	G_1	--	–	-	5	20
	16.33	G_1	--	--	.	4	100
	16.35	G_1	–	–	–	5	10
	16.39	G_1	-	–	--	5	20
	16.40	G_1	–	–	–	(5) [c]	NT
BIg or light-chain	11.3	G_1	6	7	? [f]	5	NT
	12.3	G_1	5	6	?	5	NT
	16.18	G_{2b}	5	5	5	5	NT

[a] Titer against BIg isotypes G_1, G_2, M and A, expressed as $\log_{10}$ of the reciprocal dilution giving $E_{450} > 0.100$ in the indirect ELISA (approx. $3 \times$ background E_{450}).
[b] Minimal amount of homologous BIg isotype (ng/ml) detectable in the DAS-ELISA using the relevant MCA as capturing antibody.
[c] Shallow titration curve with low E_{450} at high MCA concentration.
[d] Not tested.
[e] In addition, 11 other stable clones were obtained from fusion 16; they were not further characterized for their specificity.
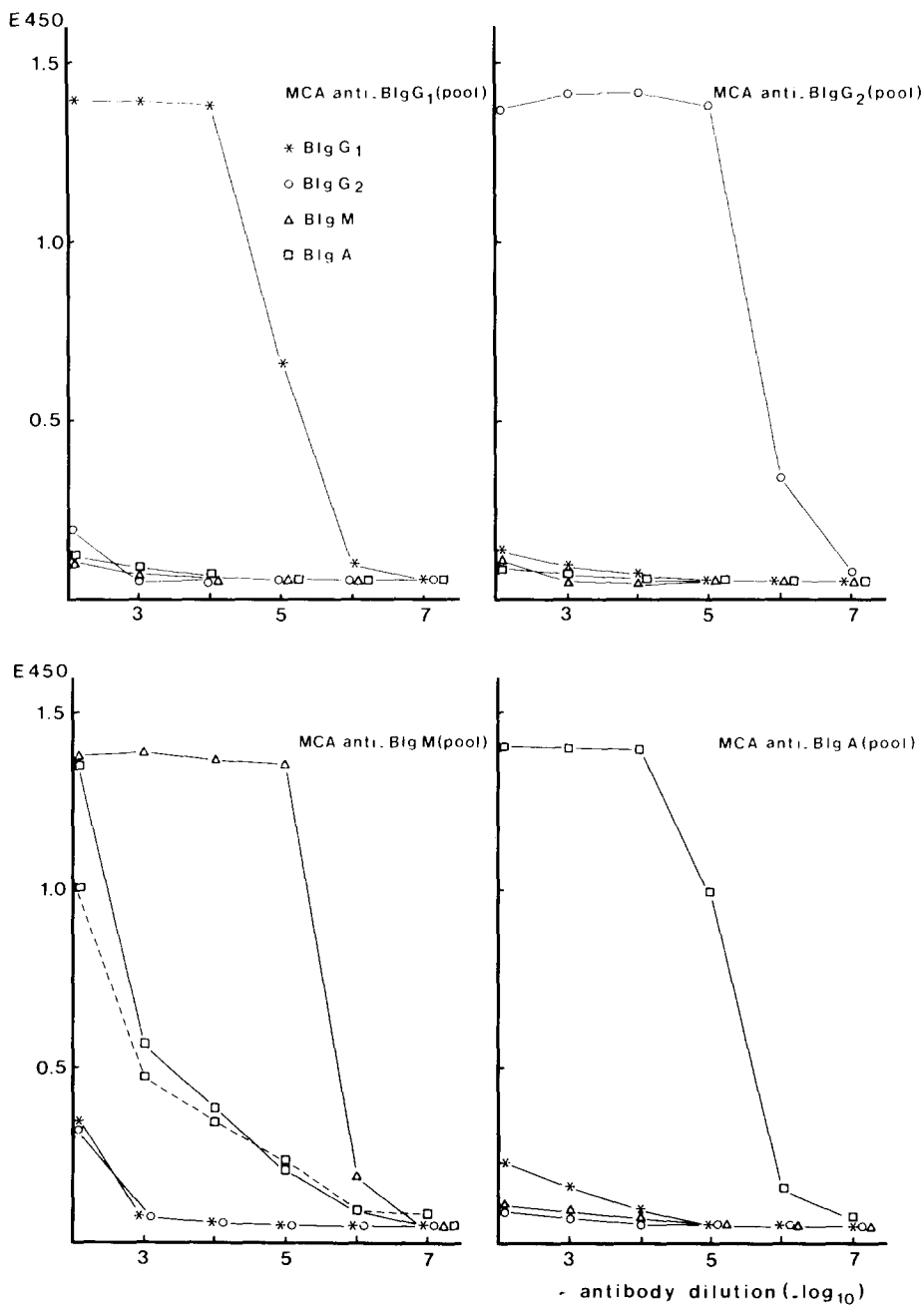[f] Reactivity depends on IgM preparation.

Fig. 2. Analysis of MCAs anti-BIg isotypes in the indirect ELISA. Pools of MCAs (Table I) were tested in 10-fold dilutions in the indirect ELISA for their reactivity with $BIgG_1$ (∗), $BIgG_2$ (○), BIgM (△), BIgA (□) or affinity purified BIgA (---□---). Individual MCAs of each pool showed the same specificity and an identical or lower titer (see Table I).

MCAs demonstrated a more complex reactivity. MCAs 11.4, 11.5 and 11.6 reacted strongly with IgG_2 but weakly with IgG_1. This latter reactivity was not reduced after additional purification of the IgG_1 preparation by passing it over an immunosorbent column of MCA anti-$BIgG_2$ (12.1). A control experiment demonstrated that this column bound IgG_2. Consequently, the cross-reactivity is not due to an IgG_2 contamination of the IgG_1 preparation. Similarly, MCAs 17.3, 17.4 and 17.11 reacted strongly with IgM but showed weak cross-reactivity with IgA, even after the IgA preparation had been purified by affinity chromatography using an immunosorbent column of MCA anti-BIgA (mixture of MCAs 16.4, 16.24, 16.33, 16.35 and 16.39) (Fig. 2). The cross-reactivity of the MCAs anti-BIgM with IgA was confirmed by the results shown in Fig. 3. MCA anti-BIgM applied as capturing antibody in a DAS-ELISA bound IgA as shown by the subsequent binding of monoclonal IgA-specific conjugate (same mixture of MCAs as above). As a control IgM was tested in the same assay and in a similar assay with a monoclonal IgM-specific conjugate instead of the anti-IgA conjugate. The results showed that IgM was bound by the capturing MCA anti-BIgM but was not recognized by the anti-IgA conjugate. Fig. 3 (lower panel) also shows that the affinity-purified IgA preparation is free of detectable IgM. In addition, it is concluded that the cross-reactivity of MCA
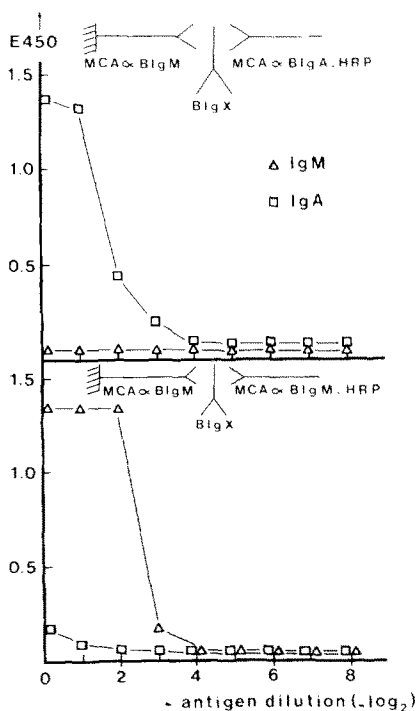

Fig. 3. Cross-reactivity of MCAs anti-BIgM with IgA in the double antibody sandwich assay. Plates were coated with an optimal concentration of MCA anti-BIgM (clone 17.3) and were incubated with 2-fold dilutions of IgM ($\triangle$) or affinity purified IgA ($\square$) (starting concentration 8 μg/ml). MCA anti-BIgA (upper panel) and MCA anti-BIgM (lower panel) were used as conjugates.

anti-BIgM with IgA is not detectable when this monoclonal is used both as capturing and conjugated antibody. Similar results were obtained with all 3 MCAs anti-BIgM.

Isotype-specific ELISAs for detection of rotavirus antibody

For the study of mucosal immunity in rotavirus infected calves, 2 kinds of isotype-specific ELISAs (IDAS and ACA, Fig. 1) were developed for the detection of antibodies against rotavirus. MCAs against BIg isotypes were used as conjugated antibodies (IDAS) or as capturing antibodies (ACA). A comparison of different MCAs used as capturing antibody in the DAS-ELISA showed that MCA 15.8, 12.5 and 16.35 were the best MCAs for detection of IgG_1, IgG_2 and IgA respectively, while MCA anti-BIgM 17.3, 17.4 and 17.11 reacted similarly (Table I). Initially, mixtures of MCAs were used as conjugates for the IDAS (Table II). Further analyses showed that conjugates of MCAs 15.8, 17.3 and 16.35 gave identical results as the mixtures with the same isotype specificity mentioned in Table II and could be used instead. However, the mixture of MCAs 12.1 and 12.5 gave slightly better results than the individual MCAs for the detection of IgG_2 anti-rotavirus antibody in the IDAS.

As a control for non-specific reactions in the IDAS and ACA test samples were also tested in the lowest test dilution (1 : 20) in an assay with a pool of rotavirus-free fecal samples (instead of the rotavirus containing sample). Non-specific reactions did not occur in the ACA but at first they were observed rather frequently in the IDAS. The reaction appeared to be stronger when serum samples from older cows were tested and increased in the order $IgA < IgG_1 < IgG_2 < IgM$. Non-specific binding of BIg was observed with uncoated ELISA plates treated with Tween 80, with antibody coated to the plates and with components from the rotavirus containing fecal sample. It could be reduced by the addition of 10% horse serum to the test samples and by lowering the incubation temperature to 4°C. Using these conditions non-specific reactions were not found with serum or fecal samples from SPF calves (age ≤ 2 months). The occurrence of non-specific reactions under these conditions with samples from older cows are still under study.

TABLE II

SELECTION OF MCAs FOR ELISA

Specificity of ELISA	Optimal capturing antibody for DAS and ACA	MCAs used as conjugate in IDAS
IgG_1	15.8	15.8 [a] + 15.17 + 15.18
IgG_2	12.5	12.1 + 12.5
IgM	17.3	17.3 + 17.4 + 17.11
IgA	16.35	16.4 + 16.24 + 16.33 + 16.35 + 16.39
All isotypes	16.18	Not applicable

[a] Individual MCAs giving the same result as the complete mixture are underlined.

Inter- and intra-isotype competition

Competition between different isotypes in the IDAS was studied by mixing samples containing antibodies against rotavirus of different isotypes followed by analysis in isotype-specific IDAS assays. The addition of increasing amounts of IgG anti-rotavirus to a fecal or serum sample containing IgM anti-rotavirus inhibits the detection of anti-rotavirus IgM (Fig. 4). Similar experiments with competing IgG_1, IgG_2, IgM or IgA anti-rotavirus showed that no inter-isotype competition could be detected in the IgG_1- and IgG_2-IDAS with the samples under study. Inter-isotype competition was not studied in the IgA-IDAS.

In ELISAs with an isotype-specific capturing antibody no inter-isotype competition can occur. However, competition for capturing antibodies does occur between antigen-specific Ig and 'normal' Ig of the same isotype. This is illustrated for IgM by the experiment shown in Fig. 5. The addition of normal IgM (as a serum sample free of rotavirus antibody) to IgM anti-rotavirus resulted in complete inhibition of IgM anti-rotavirus detection by IgM-ACA. Similar results were obtained for the IgG_1- and IgG_2-ACA. No competition was found in the IgA-ACA, most likely because the competing serum sample contained a very low amount of IgA.

Comparison of IDAS and ACA

To select an optimal isotype-specific test for the detection of rotavirus antibody, fecal, serum and colostrum samples were titrated both in IDAS and ACA. The results are given in Table III. The IDAS is to be preferred for detection of IgG_1 and
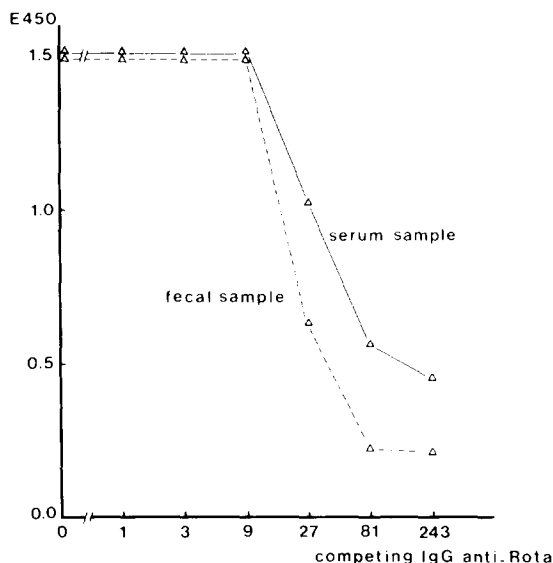

Fig. 4. Inter-isotype competition in the IgM-IDAS for rotavirus antibody. A serum sample (————) and a fecal sample (- - - - - -) containing IgM antibodies against rotavirus were mixed with 0–243 arbitrary units of IgG antibodies against rotavirus and mixtures were tested in the IgM-IDAS.
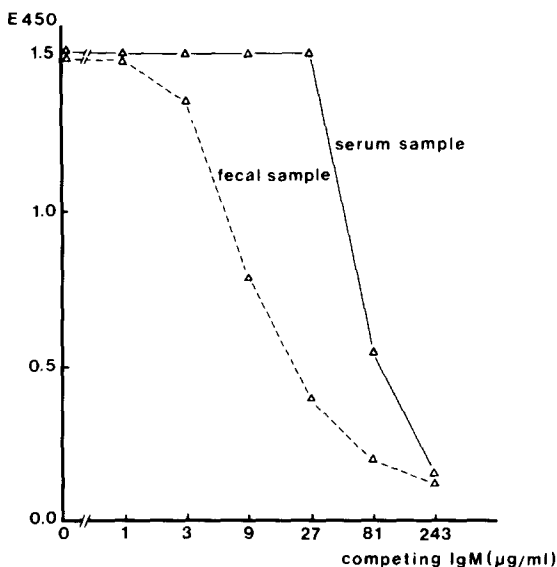
Fig. 5. Intra-isotype competition in the IgM-ACA for rotavirus antibody. A serum sample (————) and a fecal sample (- - - - - -) containing IgM antibodies against rotavirus were mixed with 0–243 $\mu g/ml$ of IgM (final concentration of competing IgM) and mixtures were tested in the IgM-ACA. The serum and the fecal sample contained approx. 5 and 23 μg of IgM/ml, respectively.

TABLE III

COMPARISON OF THE ISOTYPE-SPECIFIC IDAS AND ACA FOR THE DETECTION OF ANTIBODIES AGAINST ROTAVIRUS

Sample [a]	IgG$_1$		IgG$_2$		IgM		IgA	
	IDAS	ACA	IDAS	ACA	IDAS	ACA	IDAS	ACA
1. K35 S1	– [c]	–	–	–	–	–	–	–
2. K33 S3	640 [b]	1 280	80	1 280	160	2 560	–	40
3. K28 S6	2 560	2 560	320	5 120	–	320	–	80
4. K35 S4	2 560	1 280	80	80 [d]	20	640	20	160
5. D2457-1	–	–	–	–	–	–	–	–
6. D2318-26	–	–	–	–	–	320	–	20
7. D2316-27	–	80 [d]	–	20 [d]	80	2 560	80	1 280
8. D2466-6	2 560	80 [d]	320	80 [d]	non-spec.	1 280 [d]	20	80 [d]
9. D2359-4	5 120	80 [d]	640	80 [d]	non-spec.	1 280 [d]	20	160 [d]
10. C366-1659	640	–	320	–	non-spec.	–	–	–

[a] Samples (1–9) were obtained from different SPF calves infected orally with rotavirus: 1, preserum; 2–4, sera post-infection; 5, feces pre-infection; 6–9, feces post-infection; fecal samples 8 and 9 contain unadsorbed colostral antibody against rotavirus. Sample 10: colostrum.
[b] Reciprocal of highest dilution giving $E_{450} > 0.150$ (approx. $3 \times E_{450}$ background).
[c] Titre < 20.
[d] $E_{450} \leqslant 0.450$, also in lower dilution (intra-isotype competition).

TABLE IV

ISOTYPE-SPECIFICITY OF ELISAs FOR DETECTION OF ROTAVIRUS ANTIBODY

Sample	IgG$_1$-IDAS	IgG$_2$-IDAS	IgM-ACA	IgA-ACA
1 [a]	320 [b]	–	–	–
2	– [c]	640	–	–
3	–	–	1 280	–
4	–	–	20	640

[a] Samples no. 1 and 2: purified IgG$_1$ and IgG$_2$ respectively, containing antibody against rotavirus. Samples no. 3 and 4: fecal samples from SPF calves orally infected with rotavirus obtained 7 days post primary infection and 7 days post secondary infection respectively.

[b] Reciprocal of highest dilution giving $E_{450} > 0.150$ (approx. $3 \times E_{450}$ background).

[c] Titre < 20.

IgG$_2$ anti-rotavirus as in this test colostrum and fecal samples containing unabsorbed colostral Ig gave better results. For the detection of IgM and IgA anti-rotavirus antibody, however, the ACA is the test of choice.

Isotype-specificity

As shown above MCAs against bovine IgG$_1$, IgG$_2$ and IgA used in IDAS and ACA are monospecific in an indirect ELISA, while MCAs anti-BIgM cross-react partly with IgA. To prove isotype specificity of a particular test it is necessary to show this in the test under study. Table IV demonstrates that the IgG$_1$- and IgG$_2$-IDAS as well as the IgM- and IgA-ACA for the detection of rotavirus antibody detect only the homologous isotype, since samples with a high titer for only 1 isotype were negative in the assays for the other isotypes or reacted only very weakly (sample no. 4 in the IgM-ACA).

Discussion

Often production of MCAs against soluble antigens is frustrated by the low frequency of clones producing antibodies of the specificity wanted. In our initial attempts to produce MCAs against BIg, mouse immunoglobulins were produced by 60–70% of the growing clones but only very few clones produced antibody reacting with the specific antigen. For later fusions we adopted the immunization schedule of Stähli et al. (1980) with repetitive high doses of antigen injected shortly before fusion. Subsequently, we obtained 40–100 positive clones from each fusion. Using this approach we recently obtained from 1 spleen over 1000 clones that produced antibody with the desired specificity for swine IgG (unpublished result). In Table I and Fig. 2 we present evidence that several of the MCAs obtained react monospecifically with IgG, IgG$_1$, IgG$_2$ or IgA, while results with affinity-purified IgA prove that our anti-BIgM MCAs partially cross-react with IgA (Figs. 2 and 3). The reactivity of anti-BIgA MCAs with secretory component has not been studied yet. At present, we do not know whether the MCAs against IgM recognize different antigenic determi-

nants. At least 2 of the clones produced different antibodies since their mouse isotypes are different. This, however, does not exclude that they may react with the same antigenic determinant.

Presumably, the MCAs anti-BIgM recognize immunodominant antigenic determinant(s) of IgM which are present in a modified form in IgA, and not detectable in IgG_1 and IgG_2. Since these MCAs react with IgM isolated from serum, it is unlikely that they react with secretory component. Reaction with α-chain determinants, which may be partially hidden in IgA, cannot be excluded but seems unlikely (Brandzaeg, 1976).

Conventional antisera against bovine IgM and possibly also against IgA might contain antibodies against these cross-reactive determinant(s) and it depends on their concentration and affinity whether these antisera should be cross-absorbed with IgA or IgM respectively, to obtain monospecific antisera. The results of Butler et al. (1980) indicated that an anti-BIgM serum, which had been absorbed with IgG and fetal calf serum, showed 1–2% cross-reactivity with IgA in an ELISA. Such a low level of cross-reactivity is probably not a serious problem.

The partial cross-reactivity of MCAs anti-'$BIgG_2$' 11.4, 11.5 and 11.6 with IgG_1 confirms similar results reported by Jefferis et al. (1982) and Srikumaran et al. (1982). MCAs 11.3 and 12.3 react with IgG_1, IgG_2 and IgA, but only with some IgM preparations, and thus might recognize a light chain determinant hidden or denatured in some IgM preparations.

MCA anti-$BIgG_1$ 15.18 and MCA anti-$BIgG_2$ 12.1, which bind firmly in the indirect ELISA, are not very reactive as capturing antibody in the DAS-ELISA. This might be explained by improper coating conditions for these particular MCAs or alternatively, their affinity might be too low to use them as capturing antibody in the DAS-ELISA. The MCAs described might be interesting tools for further studies on the structure of bovine immunoglobulins.

Since MCAs bind to a single antigenic determinant the possibility exists that our MCAs specifically recognize subpopulations of Ig isotypes (e.g., IgG_{2a} and IgG_{2b}) or allotypic markers. Results up to now do not indicate that our MCAs recognize subpopulations of BIg isotypes (unpublished results). Moreover, it is unlikely that they react with allotypic markers, since our MCAs react with several isotype preparations isolated from different animals and with Ig isotypes present in sera and fecal samples from several calves used in other studies (Van Zaane et al., submitted). However, further experiments are needed to exclude these possibilities.

For the development of isotype-specific ELISAs to detect antibodies against rotavirus MCAs with optimal properties were selected. This selection (Table II) was based on their specificity and titer in the indirect ELISA and their binding characteristics in the DAS-ELISA for BIg isotype detection. The selected MCAs also appeared to be optimal reagents as conjugate, except for MCA anti-$BIgG_2$ 12.5 which demonstrated a moderate synergistic effect with MCA anti-$BIgG_2$ 12.1.

Theoretically an antibody capture assay has advantages over an indirect assay using conjugated anti-Ig isotype reagents. First, inter-isotype competition does not occur in the ACA. Second, interference with rheumatoid factors is very unlikely (Chantler and Diment, 1981). Third, anti-Ig conjugates used in the IDAS, which will

also react with non-specifically bound Ig from the test sample thus giving high backgrounds, are not used in the ACA. Our results however indicate that certain samples containing IgG_1 or IgG_2 anti-rotavirus antibody were found negative or only weakly positive in the ACA (Table III). We presume that intra-isotype competition in these samples (colostrum or feces containing unadsorbed colostral immunoglobulins) inhibited the detection of antigen-specific IgG_1 and IgG_2. With serum samples, the ACA gave equal (IgG_1) or better (IgG_2) results in comparison with the IDAS. Since it is not very practical to use different kinds of tests for different samples (serum, feces, colostrum, milk etc.) we decided to use the IDAS for detection of anti-rotavirus IgG_1 and IgG_2. On the other hand, intra-isotype competition, although it can occur (Fig. 5), did not interfere in the IgA and IgM ACA to the same extent as with IgG_1 and IgG_2 (Table III). This can be explained by the relatively short half-life of IgM and IgA (4.0 and 2.8 days respectively in comparison with 21 days for IgG (Logan et al., 1972)) which leads to high percentages of antigen-specific antibody for these isotypes. Based on theoretical grounds and the experimental results the ACA was selected for the detection of IgM and IgA anti-rotavirus.

In our opinion it is not feasible to make a reliable comparison of the sensitivity of the four isotype-specific ELISAs selected. Therefore, antibody levels can only be compared within and not between isotypes.

The isotype specificity of the selected tests is based on the specificity of the MCAs (Table I, Fig. 2) and is confirmed by the observation that samples containing high titers of antibodies against rotavirus of 1 particular isotype reacted only in the homologous assay (Table IV). Apparently, the moderate cross-reactivity of MCAs anti-IgM with IgA is only of minor influence on the isotype specificity of the IgM-ACA. Probably the presence of IgM, which reacts with a higher affinity with MCA anti-BIgM, inhibits the binding of IgA.

In conclusion, monoclonal antibodies specific for bovine Ig isotypes are now available in 'unlimited' amounts and as high titered samples. They can be successfully applied in isotype-specific ELISAs for the detection of antibodies against rotavirus. Such tests appear to be very suitable for the study of humoral mucosal immunity to rotavirus infection in calves (Van Zaane et al., submitted).

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