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Veterinary Immunology and Immunopathology, 17 (1987) 25-35
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

THE APPLICATION OF HYBRIDOMA TECHNOLOGY TO THE STUDY OF BOVINE IMMUNOGLOBULINS

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ABSTRACT

Goldsby, R.A., Srikumaran, S., Arulanandam, A., Hague, B., Ponce de Leon, F.A., Sevoian, M. and Guidry, A.J., 1987. The application of hybridoma technology to the study of bovine immunoglobulins. Vet. Immunol. Immunopathol., 17: 25-35.

Studies are described in which hybridoma technology is used to produce a variety of reagents for the characterization and manipulation of the bovine humoral immune system. Selected members of a set of murine monoclonal antibodies (MAb) specific for each of four major isotypes of bovine Ig constant regions, one specific for anti-bovine Ig constant regions as well as one specific for anti-bovine light chains are discussed. Interspecific fusion of bovine lymphocytes with the established mouse cell line, SP2/0 was used to produce a collection of stable hybridomas among which were found secretors of bovine IgG1, IgG2, IgM, IgA and bovine light chain. Interspecific fusion of SP2/0 with lymphocytes from a multiparous Holstein four days post immunization with Streptococcus agalactiae yielded MAb with specificity for the immunizing antigen. One of these hybridomas, LHRB 19.17, which displayed a particularly stable secretory phenotype, was used as an immunogen for the production of a library of murine monoclonal anti-idiotype antibodies. Competitive antigen binding analysis showed that 15 of the 24 anti-LHRB 19.17 idiotype antibodies isolated blocked the binding of the idiotype to its nominal antigen and so were candidates for evaluation as antigen mimics. Some of the ways in which monoclonal anti-idiotypes in particular, and monoclonal in general, might be of use in problems of animal disease are discussed.

INTRODUCTION

Kohler and Milstein's 1975 demonstration that monoclonal antibodies (MAb) of predefined specificity could be produced by the fusion of B-lineage cells from immunized donors with selectable myeloma cell lines provided the paradigm for an explosion of hybridoma research. In subsequent years hybridoma technology has changed the practice of applied and experimental immunology by offering the following advantages over conventional polyclonal antisera: 1) Stabilized, frozen hybridomas can be stored indefinitely thus providing a "perpetual"

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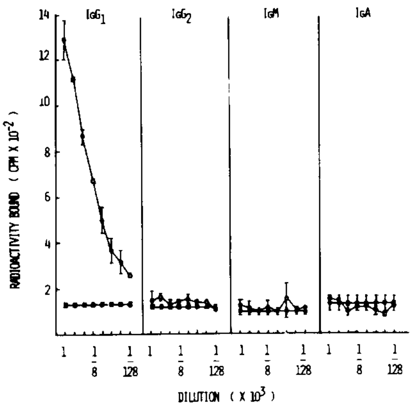
source of well-defined homogeneous MAb; 2) Large amounts (grams or kilograms) of murine MAb can be obtained with minimal investment of resources and personnel; 3) Highly complex or grossly impure antigens can be used in immunization protocols because hybridoma clones secreting MAb specific for a particular antigen can be identified and isolated; 4) Since MAb react with determinants in an "all or none" fashion, there is no need to resort to adsorption to improve specificity. Specificity is realized by cloning the hybridoma to obtain MAb without other antibodies specific for the same determinant and free of non-antibody protein; 5) Hybridoma technology provides an important route to obtain monoclonal samples of the immunoglobulin repertoire of a given species.

We will review here our progress in the use of hybridoma technology to accomplish the following: 1) Derivation of reagents for identification of bovine immunoglobulin classes and subclasses; 2) Derivation of bovine X mouse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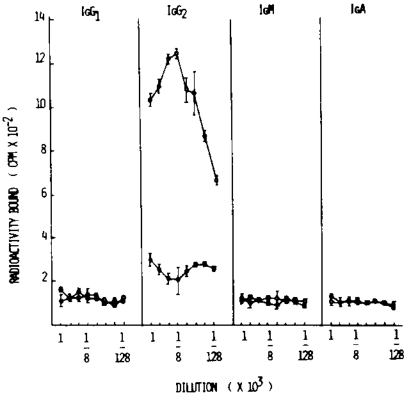
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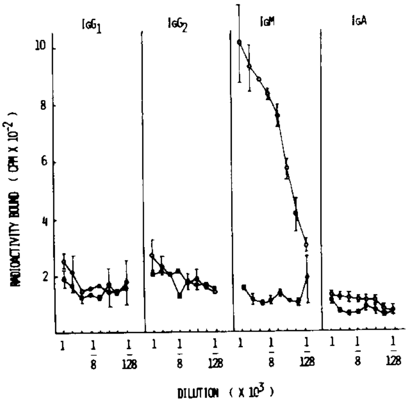
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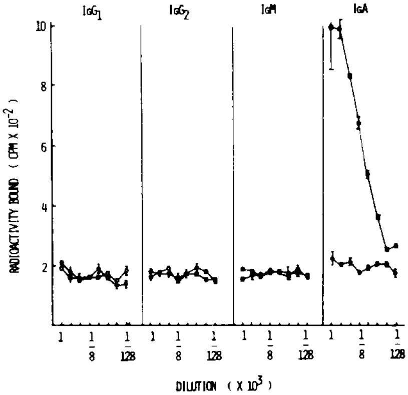
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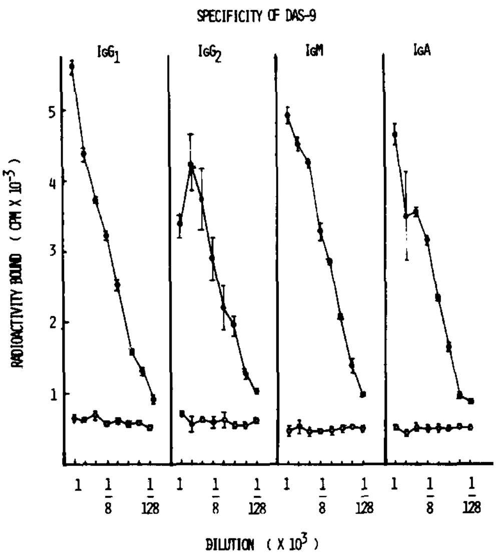

Figure 1a & 1b: Titration of affinity purified monoclonal mouse antibodies produced by the fusion of spleen cells from mice immunized with bovine Ig to the established cell line, SP 2/0 on microtiter dishes coated with the indicated bovine immunoglobulin.

hybridoma cell lines which secrete monoclonal bovine immunoglobulins; 3) the derivation of monoclonal bovine antibodies of predefined specificity; and 4) Derivation of monoclonal anti-idiotype antibodies to an antigen-specific bovine MAb.

EXPERIMENTAL RESULTS

Monoclonal antibovine immunoglobulin classes and subclasses

The ability to characterize antibody class is fundamental to the analysis of humoral immune responses. Different Ig classes perform different effector functions, may be exclusively or preferentially associated with certain immune responses or be restricted to certain compartments. Consequently, it is important to have a set of reagents of known and constant specificity for the isotypic characterization of bovine immunoglobulins. The advantages of consistency, reagent reproducibility and the specificity inherent in the hybridoma approach led us to prepare a number of class and subclass specific monoclonal reagents (Srikumaran et al., 1982). The reactivity profiles of five of the more useful members of our library of anti-bovine Ig monoclonal antibodies are shown in figures 1a and 1b. The monoclonal antibody DAS-2 is specific for bovine IgG2; DAS 17, for IgG1; DAS 7 for IgA; DAS 6 for IgM and DAS 9, specific for determinants on the light chain, reacts strongly with all bovine immunoglobulins. In practice, we use a combination of DAS 9 and DAS 10, two noncrossreacting anti-bovine light chain antibodies, in sandwich ELISA or

^{125}I solid phase assays to detect bovine immunoglobulin of any class. Class specific assays employ an immobilized anti-light chain monoclonal antibody to capture the bovine Ig and the appropriate isotype-specific MAb (Figure 1) to detect the captured Ig. These reagents were useful in the screening and characterization of the bovine Ig-secreting hybridomas described in the next section.

Bovine X mouse hybridomas which secrete monoclonal bovine immunoglobulins

Definition of bovine immunoglobulins lags far behind that of human or murine immunoglobulins (Butler, 1983). This may be due primarily to the extreme rarity of bovine myelomas. Fortunately, it has been possible to circumvent the natural paucity of bovine myelomas by resorting to hybridoma technology. Bovine spleen or lymph node cells can be fused with the aid of polyethylene glycol to the established mouse myeloma cell line SP 2/0 to produce interspecific hybridomas which secrete bovine Ig (Srikumaran et al., 1983a, 1983b). Although chromosome loss is expected in interspecific hybrids we and others (Nowinski et al., 1980) have found that aggressive techniques of selection and cloning permit isolation of the small number of stable antibody-secreting hybrids. Srikumaran et al. (1984) found that fusion of bovine lymphoid cells with SP 2/0 resulted in hybridomas in 353 wells, 49 (13.8%) secreted bovine Ig and on subcloning 24 of these retained the secretory phenotype. Thus, the yield of secreting hybridomas obtained from this fusion was 6.8%, about 1 in 20.

We have used this technology to provide monoclonal reference immunoglobulins for the major classes of bovine Ig. In contrast to polyclonal standards monoclonal Igs provide a single defined molecular species reproducible in quantity and widely available from frozen cell lines. The superiority of monoclonal standards is evident in the fields of murine and human immunology which for years have chosen monoclonal rather than polyclonal immunoglobulin standards.

A collection of hybridomas which secrete bovine IgA, IgG1, IgG2, IgM and light chain has been obtained (Table 1). While demonstrating the feasibility of generating Ig-secreting bovine X mouse hybridomas the library so far is heavily skewed toward IgM. We seek to produce a wider representation of other Ig classes so that several monoclonal examples of each isotype and subisotype can be made available.

Derivation of monoclonal bovine antibodies of predefined specificity

The derivation of hybridomas which secrete MAb of predefined specificity is routine in the murine system and is well advanced in the human system. However, there are only two examples of the successful application of this technology to systems of veterinary interest. One is the derivation of a monoclonal bovine anti-DNP (Srikumaran et al., 1983b) and a report of a set of bovine hybridomas which react with bovine enteric coronavirus (Raybould et al., 1985). The latter report established the feasibility of deriving and stabilizing hybridomas which secrete bovine antibody specific for viruses. We have derived monoclonal bovine antibodies specific for Streptococcus agalactiae, an agent of mastitis (Table 1). Antigen-stimulated lymphocytes for the production of hybridomas were obtained from a multiparous Holstein cow immunized with heat killed S. agalactiae by injecting 5 X 10^8 bacteria i.v. and 5 X 10^8 s.c. around each quarter of the udder. Mesenteric and supramammary lymph node cells were harvested 4 days after immunization and fused to SP 2/0 as outlined in Srikumaran et al. (1983b).

A bovine IgM-secreting hybridoma, LHRB 19.17 recognized a heat stable (15 minutes at $100^{o}C$) S. agalactiae determinant (Figure 2). This is one of the most stable secretors, intraspecific or interspecific that we have encountered. One month post stabilization, LHRB 19.17 was subcloned and all of the 55 resulting clones secreted antibody. Examination of the karyotype of this hybridoma revealed that it retains 12 of the original cohort of 60 bovine chromosomes and so, by inference, would be expected to express stably a number of bovine phenotypes distinct from Ig. These results and the success of

TABLE 1

Library of monoclonal bovine immunoglobins.

MAB (isotype)	Binds to	MAB (isotype)	Binds to
LHRB-1 (IgG_1)	Unknown	LHRB-115.13 (IgM)	Unknown
LHRB-2 (IgG_2)	Unknown	LHRB-115.16 (IgM)	Unknown
LHRB-3 (IgM)	Unknown	LHRB-19.2 (IgM)	Unknown
LHRB-4 (IgG_1)	Unknown	LHRB-19.4 (IgM)	Unknown
LHRB-5 (IgG_1)	Unknown	LHRB-19.5 (IgM)	Unknown
LHRB-6 (Lt chain*)	Unknown	LHRB-114.3 (IgM)	Unknown
LHRB-8 (IgM)	Unknown	LHRB-114.7 (IgA)	Unknown
LHRB-9 (IgM)	Unknown	LHRB-114.21 (IgM)	Unknown
LHRB-115.1 (IgM)	Unknown	LHRB-114.45 (IgM)	Unknown
LHRB-115.8 (IgM)	Unknown	LHRB-114.53 (IgM)	Unknown
LHRB-115.9 (IgM)	Unknown	LHRB-612.44 (IgM)	Unknown

Hybridomas secreting bovine monoclonal antibodies with relevant to animal disease:

LHRB-114.4	(IgM)	Strep. agalactia & E. coli
LHRB-115.1	(IgM)	Strep. agalactia
LHRB-1917	(IgM)	Strep. agalactia

*This hybridoma initially secreted IgM and then apparently, lost the Mu chain synthetic ability, but continued to secrete the light chain.

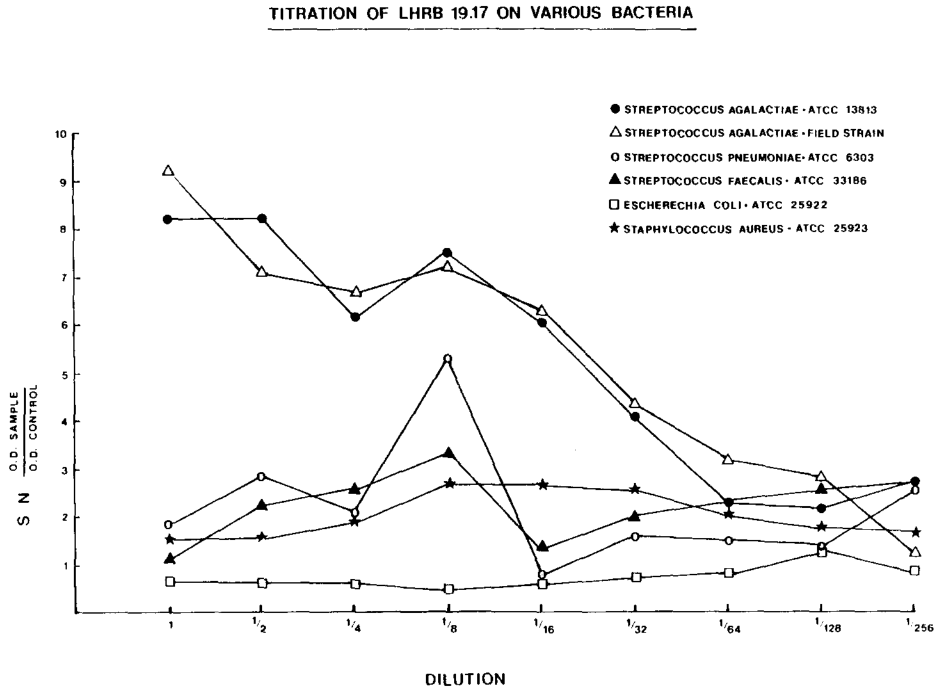
TITRATION OF LHRB 19.17 ON VARIOUS BACTERIA

Figure 2: Specificity of interspecific monoclonal antibody of LHRB 19.17. 50 ul aliquots of the indicated dilutions of culture fluid were tested by solid phase ELISA on PVC plates to which the indicated bacterial strains had been attached by glutaraldehyde fixation. S/N is the signal to noise ratio.

Raybould et al. (1985) in raising bovine MAb against bovine enteric corona virus suggest that interspecific fusion may be generally useful for the derivation of bovine MAbs of any desired specificity. Clearly, the preparation of useful bovine MAb need not wait until a suitable bovine B lymphoblastoid cell line has been found, made HAT sensitive, and shown to produce antibody-secreting hybridomas.

Derivation of monoclonal anti-idiotype antibodies to an antigen-specific bovine monoclonal antibody

Jerne (1974) proposed a network theory of the immune system in which idiotypes and anti-idiotypes play a central regulatory role. A necessary concommittant of this theory is that the binding site (idiotope) of an antibody (AB 1) which is complementary to an antigen (AG 1) will be recognized by an anti-idiotype (AB 2). In some instances, the binding site of AB 2 will bear epitopes that are conformationally quite similar to the epitopes of the original antigen, AG 1. A number of interesting demonstrations of the capacity of AB 2 to mimic some of the biological effects of the antigen, AG 1, have been described and have led to consideration of anti-idiotypes as alternatives to antigen for the induction of immunity. Early work, employing a variety of antigens, demonstrated that the administration of anti-idiotype can lead to suppression of normally dominant idiotopes (Hart et al., 1972; Strayer et al., 1974) or to induction of the corresponding idiotypes (Trenker and Riblet, 1975). Also, in some systems it became apparent that whether the outcome of treatment with anti-idiotype was induction or suppression was dependent on dose and antibody class (Eichman, 1974). Finally the capacity of anti-idiotypes to mimic the biological effects of the molecules used to elicit AB 1 are not limited to the immune system. For example, Serge and Peterson (1978) have shown that anti-idiotype antibodies raised against bovine anti-insulin inhibited the binding of ^{125}I insulin to the receptors of rat epididymal fat cells. Particularly striking was the observation that the interaction of this anti-idiotype with rat thymocytes stimulated their uptake of alpha-aminoisobutyric acid thereby mimicking the action of insulin. Clearly, anti-idiotypic antibodies sometimes mimic critical structural features of the nominal or external antigen and so can act as immunogens which are alternative to conventional antigens. To develop the reagents for a study of idiotype/anti-idiotype interactions in a system of veterinary interest we explored the feasibility of raising monoclonal anti-idiotype antibodies to an antigen-specific monoclonal bovine immunoglobulin. Toward this end mouse monoclonal anti-idiotype antibodies were prepared as described below against LHRB 19.17 which is specific for S. agalactiae.

Balb/c mice (8-16 wk old) were primed by i.p. injection of 100-200 ug of affinity purified, S. agalactiae-reactive bovine idiotype precipitated on alum and emulsified in complete Freund's adjuvant. Three weeks later the mice received by i.p. injection 118 ug of the affinity purified bovine idiotype emulsified in incomplete Freund's adjuvant. Five days later spleen cells were harvested and fused to the mouse cell line SP 2/0 (Shulman et al., 1978) as described in Srikumaran et al. (1983b). The identification of the resulting mouse hybridomas which secreted monoclonal antibodies specific for the immunizing idiotype was accomlished by screening against the following antigens: BSA (control for nonspecific binding); LHRB 8, a monoclonal bovine IgM that does not react with S. agalactiae (identifies those antibodies which are merely isotype specific) and the immunizing antigen, LHRB 19.17. This procedure resulted in the identification of a number of idiotype specific antibodies (Figure 3).

In order to determine if any of the idiotype specific antibodies were candidate S. agalactiae antigen mimics, a determination was made of their

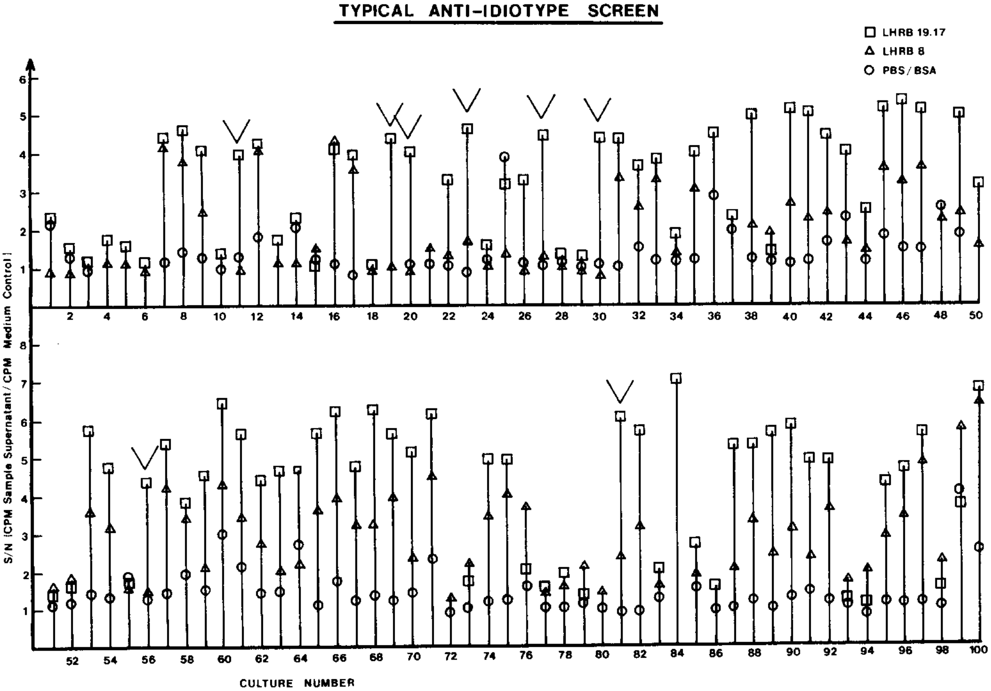

Figure 3: Screen of mouse hybridoma for production of bovine-specific anti-idiotype S. agalactiae-specific. Supernatants were screened by a solid phase ELISA assay using three sets of polyvinyl chloride microtiter plates as follows: Set 1) 20 ul of a 20 ug/ml solution of LHRB 19.17 a S. agalactiae-reactive monoclonal bovine IgM; set 2) 20 ul of a 20 ug/ml solution of LHRB 8, a monoclonal bovine IgM which has no reactivity with S. agalactiae; and set 3) 20 ul of a 100 ug/ml solution of bovine serum albumin (BSA). Hybridomas producing antibody which reacts with the monoclonal immunizing idiotype, LHRB 19.17, but not BSA or LHRB 8 are subcloned. Such clones are indicated V.

capacity to block the binding of the idiotype (LHRB 19.17) to its nominal antigen, S. agalactiae. This determination was made using wells of polyvinyl chloride microtitration plates coated with 50 ul of freshly grown S. agalactiae which had been washed twice in PBS and adjusted to a concentration of 5 X 10^8 cells/ml. The plates were centrifuged (1500 rpm for 15 minutes), the supernatant removed and the bacteria fixed by addition at 4^oC of 0.25% glutaraldehyde in PAS with subsequent incubation (15 minutes at 4^oC) and gentle washing 3X with PBS> Wells were then filled with PBS containing 0.1% BSA and 0.1% NaN_3 (PBS/BSA) and stored at 4^oC until use. Plates were washed 3X with PBS prior to use. Blocking experiments were performed by incubating 160 ul of an anti-idiotype supernatant or dilutions of control ascites fluid or serum with 40 ul of a 6 ug/ml solution of the S. agalactiae-specific monoclonal antibody LHRB 19.17 at 4°C for 24 hours. Uninhibited controls were constituted by incubation of LHRB 19.17 with supernatant or dilutions of control S. agalactiae antibody-free ascites fluid or serum as appropriate. Following the incubation, 40 ul of the mixture was transferred to a washed S. agalactiae-coated plate, incubated at 4°C overnight and then washed 3X with PBS/BSA, incubated for 1 hour with 20K cpm of ^{125}I-labelled DAS 9 (monoclonal, bovine L chain-specific) washed 3X with PBS/BSA and counted to determine the amount of LHRB 19.17 bound. The percent inhibition of LHRB 19.17 binding to S. agalactiae by anti-idiotype hybridomas is calculated as follows: a-b/a X 100 where a=CPM of LHRB 19.17 in the presence of negative control; b=CPM of LHRB 19.17 in the presence of the anti-LHRB 19.17 idiotype.

Table 2 shows the spectrum of antigen blocking we have encountered using our library of anti-idiotypes and Table 3 provides an overall summary of our experience in deriving anti LHRB 19.17 hybridomas. Of the 24 idiotype specific clones isolated, 15 (60%) are antigen blocking. This is an important consideration because any anti-idiotype that mimics the nominal antigen of the idiotype will compete with that antigen for the antigen binding site. However,

TABLE 2

Blocking by various mouse monoclonal anti-idiotypic antibodies of S. agalactiae-specific monoclonal (LHRB 19.17, mouse X bovine) binding to S. agalactiae antigen.

Anti idiotypic hybridoma antibody	% Inhibition[1]	CPM LHRB 19.17 +LHR.AID / CPM LHRB 19.17
LHR.AID.L	93.1%	366/5278
LHR.AID.56	65.7%	1812/5278
LHR.AID.49	90.5%	504/5278
LHR.AID.81	92.2%	412/5278
LHR.AID.40	62.5%	1980/5278
LHR.AID.20	92%	424/5278
LHR.AID.59	93.4%	346/5278
LHR.AID.23	92.2%	411/5278

[1] Inhibition of binding ^{125}I-labelled monoclonal anti-bovine L chain (secondary AB) to bovine monoclonal anti-S. agalactiae (LHRB 19.17) following preincubation of LHRB 19.17 with various monoclonal anti-LHRB 19.17 idiotypes prior to its application to S. agalactiae-evolved microtitre wells.

TABLE 3

Summary of the derivation of anti-LHRB 19.17 idiotype hybridomas

	Total Screened	Bovine Ig Reacting	LHRB 19.17 Idiotype Specific	Antigen Blocking
Number of hybridomas	132	73	24	15

for a variety of reasons, not all antigen blocking anti-idiotypes mimic the immunogenic properties of antigen. Therefore, having established that many of our anti-idiotypes meet the necessary condition of antigen blocking, we await the assessment of their capacity to elicit an anti-*S. agalactiae* response to determine which are true antigen mimics.

DISCUSSION

Monoclonal antibodies in general, and those of bovine origin in particular, might be of use in problems of animal disease. Passively administered MAbs have been shown to be useful for the therapy or prevention of disease:: 1) Bluetongue virus neutralizing MAbs protect mice and sheep (Letchworth et al., 1983); 2) MAb against herpes simplex virus glycoproteins protects mice against virus-induced neurological disease (Dix et al., 1981); and 3) A murine MAb against *E. coli* protects calves against enteric colibacillosis (Sherman et al., 1983) and is commercially successful. However, all of these applications have involved mouse MAb. For the passive immunotherapy of veterinary species homologous antibodies in which the effector function-mediating C regions of the Ig are most closely matched to the effector systems of the host would be advantageous. Specifically, in evaluation of mechanisms of humoral immunity such as complement fixation, antibody-mediated opsonisation Ig class-related function, Ig-halflives and tissue distribution there are clear advantages to the use of homogeneous monoclonal antibodies rather than polyclonal antibodies which differ in affinity and epitope specificity. Finally, demonstrations of the effectiveness of anti-idiotypes as alternative vaccines have been made in several model systems (Sacks et al., 1982; McNamara et al., 1984; Sharpe et al., 1984; Uytdehaag and Oosterhaus, 1985). Homogeneous idiotype is an excellent target for the preparation and characterization of anti-idiotype antibodies and similarly, for the reasons previously mentioned, hybridomas are the preferred source of anti-idiotype antibodies.

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