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Monoclonal Antibodies in Veterinary Medicine

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Introduction

In 1975 Köhler and Milstein discovered that they could fuse mouse myeloma cells with spleen cells (lymphocytes) of immunized mice, to obtain cell lines that produced antibodies with a single specificity characteristic of individual lymphocytes from immunized mice, but with the myeloma cells' ability to multiply continuously (Köhler and Milstein, 1975). The resultant hybrid cell or hybridoma expresses both the lymphocyte's property of specific antibody production and the immortal character of the myeloma cell, allowing it to be grown in tissue culture. The hybridoma produced is monoclonal because the cells in culture originated from the division of one cell. The hybridoma growing in tissue culture thus produces a monoclonal antibody. A monoclonal antibody is chemically, physically and immunologically a distinct molecule specifically produced by all the cells of a clone.

The hybridoma can be injected into histocompatible mice to produce ascites cell tumours and the resultant ascites fluid becomes a source of high-titred monoclonal antibody. These hybridoma cells can be frozen in liquid nitrogen for indefinite periods. When needed, the cells can be thawed and recultured for continued production of the identical specific antibody. The monoclonal hybridoma secreting anti-sheep red blood cell antibodies which Köhler and Milstein produced in 1975 would, if available now, produce today—over ten years later—antibody identical in all respects to that originally described. Once established, a hybridoma cell line continues to produce identical antibody molecules. Thus we have a technology for production of an unlimited standardized supply of a highly specific antibody that can be used world wide in antibody-mediated reactions.

Abbreviations: HAT, hypoxanthine–aminopterin–thymidine medium; HGPRT, hypoxanthine–guanine phosphoribosyltransferase; PEG, polyethylene glycol.

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A major characteristic of the immune response to an antigen, usually a foreign substance, is its heterogeneity, with many different antibodies usually being produced in response to each antigen because many lymphocytes produce antibody in response to antigenic stimulation, each line of lymphocytes producing a slightly different antibody. Immune serum or polyclonal antibodies raised in animals for diagnostic purposes are thus invariably a mixture of antibodies. An immune serum may be a mixture of isotypes of antibody. This can complicate their use as diagnostic reagents. The heterogeneity of polyclonal antibodies may lead to unexplained cross-reactivity in serological assays.

A monoclonal antibody is an antibody directed against one antigenic determinant or epitope of an antigen. It is a single isotype. By testing a number of different clones it is possible to select those producing monoclonal antibody of an appropriate specificity and isotype suitable for a particular serological assay.

Different animals make different antibodies against each determinant. There is no guarantee whatsoever of reproducibility from animal to animal in polyclonal serum production. If the animals are killed after a production lot there is a limited supply of reagent as the next animal will produce antibodies of different specificity, affinity and isotype. Similarly, if animals are repeatedly reimmunized and bled there is a continuous quantitative change in the quality of the resultant antibody from the same animal as new populations of lymphocytes respond to the antigen.

The homogeneity, reproducibility and permanent availability of monoclonal antibodies are the attributes arousing the great interest and research fervour in this quickly developing field.

Production of monoclonal antibody

Procedures for monoclonal antibody production have been outlined in several publications (Oi and Herzenberg, 1979; Fazekas de St Groth and Scheidegger, 1980; Galfré and Milstein, 1981; Goding, 1982; Løvberg, 1982; Bastin, Kirkley and McMichael, 1982; and Prabhakar, Haspel and Notkins, 1984). It should be noted that methods vary considerably. The following is a brief outline of the methods currently used in this laboratory, adapted from the protocol of Kennett *et al.* (1978). Although this is the traditional procedure, many modifications have been described. A schematic presentation of the procedure is given in *Figure 1*.

PRINCIPLES OF MONOCLONAL ANTIBODY PRODUCTION

Antigens introduced into animals generally result in the stimulation of lymphocytes, some of which produce antibody. Each lymphocyte produces only one type of antibody although the isotype may change; however, each lymphocyte line produces antibody that varies in (for example) specificity. Thus a polyclonal antibody response is a mixture of antibody molecules which may be similar in specificity and physicochemical characteristics but

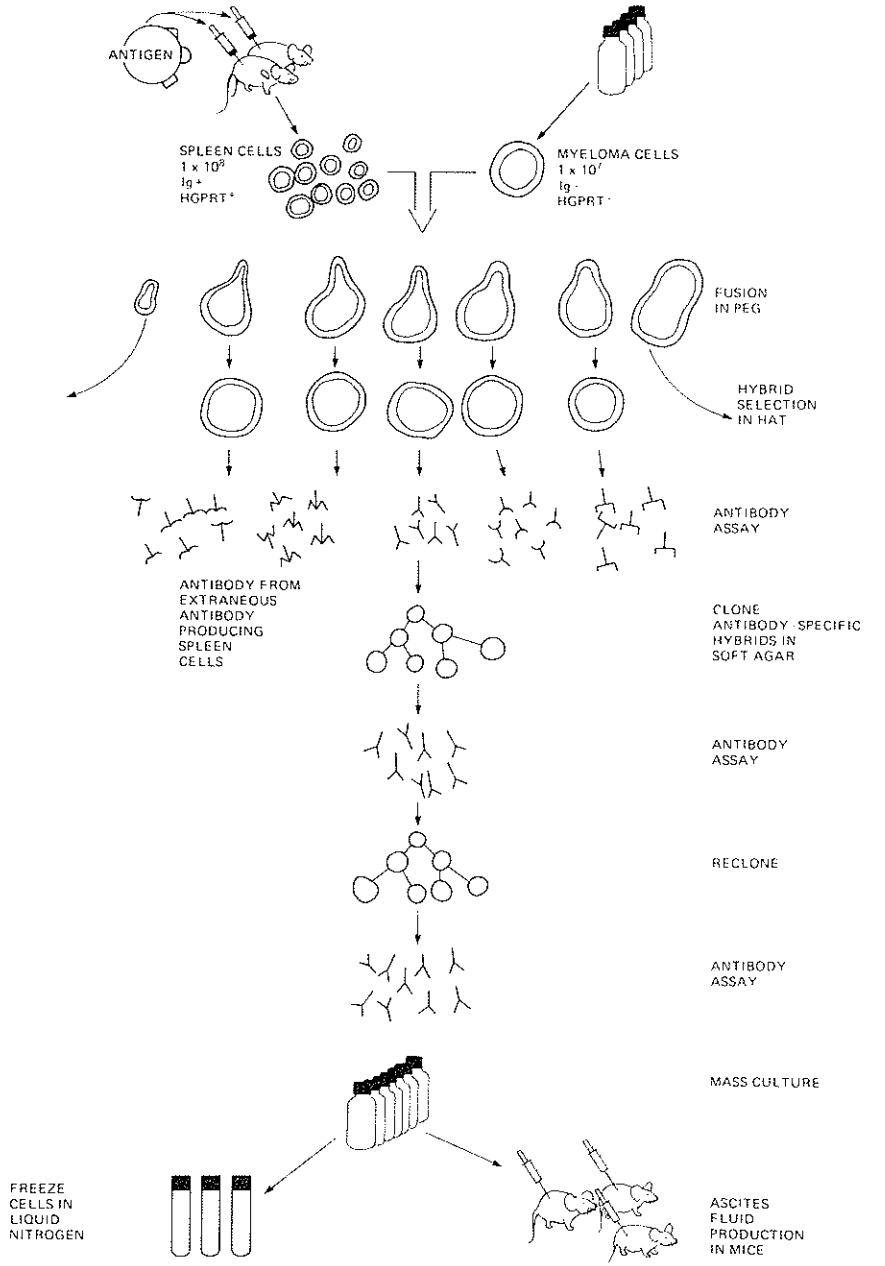

Figure 1. Schematic presentation of the essential steps in production of monoclonal antibodies in the mouse. Detailed explanations of each step are in the text.

not identical. Lymphocytes, if removed from the animal, die quickly in culture. Lymphoma (myeloma) cells on the other hand multiply indefinitely in culture but it is rare to find a line that produces antibody of any known specificity. Strains of lymphoma cells can be selected or obtained that produce no immunoglobulin-like molecules and which are deficient in hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) and are therefore unable to survive in selective media (HAT, containing hypoxanthine, aminopterin and thymidine). Fusion of the antigen-stimulated lymphocytes with lymphoma cell line results in hybrid cells which survive in HAT medium, having inherited hypoxanthine phosphoribosyltransferase from the lymphocyte and the ability to multiply indefinitely from the lymphoma cell. Both populations of 'parent' cells will die quickly. Some of the hybrid cells will produce antibody of the desired specificity and isotype, and subsequent cloning and recloning will result in a cell line that produces monoclonal antibody, i.e. a hybridoma.

Preservation of hybridoma cells

Hybridoma cell lines are most commonly preserved by freezing in liquid nitrogen. When freezing, hybridoma cells must be in the log-phase of growth. Cells are added to dimethyl sulphoxide, frozen at −70°C overnight and transferred to liquid nitrogen the following day.

The same procedure is used for freezing murine myeloma cell lines (Bastin, Kirkley and McMichael, 1982).

Ascites fluid production

Male balb/c mice 6 weeks old are injected intraperitoneally with the mineral oil pristane (2,6,10,14-tetramethylpentadecane). Two weeks later hybridoma cells from selected clones are injected intraperitoneally into the pristane-treated mice. One week to 10 days after injection the peritoneal cavities will appear swollen and an 18 gauge needle is inserted into the cavity to collect ascites fluid in heparin. Ascites fluid is collected three times in a 6-day period, after which the mouse is killed (Brodeur and Tsang, 1986).

Purification of monoclonal antibody from ascites fluid

The ascites fluid is centrifuged (1000 × *g* for 15 min) to remove cells and fibrin clots and is then centrifuged again (52 000 × *g* for 10 min) to remove lipids and small fibrin clots. Antibodies are then precipitated from the supernate by the addition of an equal volume of saturated ammonium sulphate $(NH_4)_2SO_4$ to the clarified ascites fluid. The resulting precipitate is dissolved in physiological saline and dialysed against physiological saline for 12–16 hours in the cold. The dialysate contains about 80% antibody which may be further purified or used directly.

DISCUSSION OF PROCEDURES USED IN MONOCLONAL ANTIBODY PRODUCTION

Myeloma cell lines

A number of different murine myeloma cell lines have been successfully used as fusion partners in hybridoma techniques (Westerwoudt, 1985). The attributes of an ideal myeloma cell line include rapid growth, lack of secretion, death in HAT and resistance to 6-thioguanine.

The Sp2/0 cell line does not secrete or internally produce mouse immunoglobulin molecules or their fragments (Shulman, Wilde and Köhler, 1978). When Sp2/0 is used as the parent myeloma in a fusion, the hybrid cell produces only one antibody molecule—that of the spleen cell donor. (The use of a secreting myeloma cell line as a fusion partner would result in only about one-sixteenth of the immunoglobulin produced being antigen-specific antibody.)

The Sp2/0 cell line is resistant to 6-thioguanine and dies in HAT selection medium. The mouse myeloma line has been genetically manipulated so that it lacks the enzyme hypoxanthine–guanine phosphoribosyltransferase (HGPRT) and passage continuously in the presence of 6-thioguanine selects against revertants. The lack of HGPRT is responsible for selection of the hybrid in HAT medium (Littlefield, 1964).

The unfused myeloma cells must be killed to allow the hybrid cell to grow. The myeloma cells lacking HGPRT cannot use exogenous hypoxanthine to synthesize purines and, when grown in the presence of aminopterin which blocks endogenous synthesis of purines, these cells die. Unfused spleen cells die in culture after a few replications. Spleen cells have the enzyme HGPRT: the hybrid, myeloma–spleen, therefore can use the exogenous hypoxanthine to synthesize purines and thus survives in the HAT selection medium.

Non-murine hybridomas

Rat. Production of rat–rat hybridomas may be preferable for monoclonal antibody production. The amount of serum and ascites fluid is ten times more in tumour-bearing rats than mice (Galfré, Milstein and Wright, 1979). For large-scale production it would be cheaper, although the generation time is longer. The LOU/Ws1 rat strain is used with the rat myeloma cell line 210 RCY3- Ag 1.2.3, resistant to 8-azaguanine as the myeloma fusion partner.

Human. Human–human hybridoma cell lines have been developed but with limited success. Human cell hybridomas are aneuploid and tend to lose chromosomes. Cell viability is variable and this avenue of immunoglobulin production has not been as successful as mouse–mouse hybridomas or rat–rat hybridomas. Human hybridomas obviously cannot be grown as ascites tumours.

Cattle. The occurrence of myelomas in cattle is rare. There has been one report in the literature but no further investigation was pursued (Rodkey and Kimmel, 1972).

Interspecies. Cross-species fusions have been successful (Srikumran, Guidry and Goldsby, 1984). Fusions using murine myeloma cells (Sp2/0) and bovine lymph node cells from immunized animals or blasts from LPS-stimulated lymph node cell cultures produced hybridomas secreting bovine immunoglobulins (Hague *et al.*, 1985).

Murine–porcine fusions have also been successful in producing hybridomas secreting porcine immunoglobulins (Raybould *et al.*, 1984).

Caution should be exercised when working with murine myeloma lines and murine hybridomas as they are known to carry retroviruses, both A-oncorna viruses as well as C-oncorna viruses having been reported (Bartal *et al.*, 1982; Weis, 1982; Rudolph, Karsten and Micheel, 1983).

Immunization

In vivo stimulation of spleen cells for monoclonal antibody production has been routinely accomplished by immunizing an inbred strain of rodent. Immunization protocols differ depending on the antigen used. An advantage of the monoclonal antibody production technique is that a pure antigen is not required for *in vivo* immunization; however, purified antigen is required for hybridoma screening. Long immunization protocols, with frequent injections, do not necessarily guarantee better results. Once the desired immune response is manifest in the mouse, one last injection of the immunogen is given intravenously in the tail and the mouse is killed three days later. The last injection ensures that the highest possible number of antigen-specific B-lymphocytes are dividing at the time of fusion.

In vitro immunization has also been successful in monoclonal antibody production (Reading, 1982; Borrebaeck, 1983). This method of immunization may be preferable to *in vivo* immunization for the following reasons:

1. A shorter immunization period is required;
2. It allows more control over the concentration of antigen used;
3. Cultures can be tested easily for optimum sensitization;
4. Amounts of antigen used would be less and, for expensive antigens, this would be an advantage;
5. Self-antigens or antigens pathogenic to the animal being immunized can be better dealt with using *in vitro* cultures.

However, *in vitro* immunization produces a majority of IgM isotypes. IgG isotypes may be more desirable for application in antibody-mediated reactions. Initial immunization *in vivo* with a semi-pure antigen and subsequent immunization *in vitro* with pure antigen may increase the ratio of IgG to IgM isotypes.

Fusion

It is important to carry out the fusion procedure with both myeloma and spleen cells that are actively dividing. The myeloma cell line is fed nutrients in such a fashion that it is in the log-phase of growth at the time of fusion, and the final intravenous antigen administration to the immunized mouse is three days before fusion to ensure that B-lymphocytes are at the correct stage of differentiation for optimum fusion to occur.

Fusion of the cells is accomplished by incubation of the myeloma–spleen mixture with 50% PEG 1450 for a brief period. Fusion using polyethylene glycol (PEG), a surface-active agent, was introduced by Pontecorvo (1976). Polyethylene glycols of different molecular weights (1000–4000 daltons) have been used for fusion; however, PEG with a molecular weight of 1450 (Kodak) gives more hybrid clones (Lane, Crissman and Lachman, 1984).

Köhler and Milstein (1975) first used Sendai virus as the fusing agent. More recently, fusion has been accomplished by short electric field pulses of high intensity (Zimmerman, 1983; *see also* Chapter 2 of this volume). It has proved to be a very efficient method of hybridization but equipment is expensive and asepsis is a problem.

Ascites fluid production

While hybridomas produce up to 100 μg/ml of specific antibody in tissue culture, the amount of antibody in ascites fluid is 100–200 times more concentrated. From 10 to 15 ml of ascites fluid can be obtained from one mouse.

The titre in ascites fluid is 100–1000 times higher than can be obtained from an immunized animal. One mouse can produce as much antibody as an immunized rabbit, and the antibody produced in the mouse is more concentrated and more easily purified than the polyclonal antibody generated in the larger animal.

Potter, Pumphrey and Walters (1972) showed that, in balb/c mice, pre-injection with mineral oil enhanced tumour formation. Pristane is injected 14 days before the hybridoma into male balb/c mice between 43 and 75 days old to obtain ascites fluid in the largest volume and containing the highest yield of specific monoclonal antibody (Brodeur, Tsang and Larose, 1984).

In vivo production of rat monoclonals in histocompatible rats is sometimes difficult (Hirsch *et al.*, 1985). Hybridomas do not grow when injected intraperitoneally in histocompatible rats. However, after initial passage through the liver via intravenous injection of histocompatible rats, the intraperitoneal injection of liver homogenate produces ascites fluid containing the specific monoclonal antibody.

In vitro production of monoclonal antibody in mass culture is also used (Schönherr and Houwink, 1984). Monoclonal antibody from tissue culture fluid may be preferable to monoclonal antibody from ascites fluid as the latter may be contaminated with host mouse immunoglobulins whereas the

former is truly monoclonal if serum-free media has been used to grow the hybridoma. Two problems arise when using tissue culture fluid for monoclonal antibody production: (1) removal of the serum in culture medium for reasons of cost reduction and purification and standardization of antibody; (2) the search for methods which allow scale-up to achieve a high concentration of monoclonal antibody.

Purification of monoclonal antibody

Affinity chromatography using protein-A (an immunoglobulin-binding protein derived from certain strains of *Staphylococcus aureus*) is used to separate subclasses of mouse IgG. Mouse immunoglobulins IgG_{2a}, IgG_{2b}, IgG_3, some IgG_1 and some IgM will bind to protein-A (Goding, 1978).

Ey, Prowse and Jenkin (1978) have described the use of various buffers to elute different classes of mouse immunoglobulins from protein-A Sepharose columns.

More recently, ion-exchange high-performance liquid chromatography (HPLC) has been used to isolate monoclonal antibody activity (Gemski *et al.*, 1985a). The chromatographs show excellent resolution of the IgG and the transferrin and albumin constituents which are the main contaminants of mouse ascites fluid. The same procedures that are used for isolation of monoclonal antibody from ascites fluid can be applied to tissue culture supernatants from hybridoma culture in serum-free media (Gemski *et al.*, 1985b).

Uses of monoclonal antibodies

There are three general areas in which monoclonal antibodies are used in human and veterinary medicine. These areas are: (1) immunodiagnostic reagents either as a tool to demonstrate the causative agent(s) directly in tissues or body fluids or as a reagent used in indirect diagnostics such as serological detection of antibodies to the causative agent(s); (2) for experimental purposes ranging from molecular dissection of antigenic epitopes to monoclonal anti-idiotype antibody being utilized as a vaccine to induce protective immunity and (3) immunoprophylaxis or immunotherapeutics applied to infectious diseases or as a vehicle for delivering toxic substances to, for example, tumours or as a tool to identify, locate and target tumours.

Immunodiagnostic reagents

ANTIGEN DETECTION OF INFECTIOUS AGENTS

Considerable advances have been made in the detection of infectious agents (or their antigens) directly in tissue or body-fluid specimens with the development of monoclonal antibodies of various specificities. Perhaps the greatest amount of work has focused on viruses because of the difficulties of viral culture. Many of these achievements have been reviewed recently (Carter

and ter Meulen, 1984; van Zaane, 1984; Massey and Schochetman, 1985). Monoclonal antibodies have been developed against a large number of viruses and, although complete listing is beyond the scope of this review, some notable examples are rabies virus (Koprowski and Wiktor, 1980), foot-and-mouth disease virus (McCullough and Butcher, 1982), Newcastle disease virus (Russell and Alexander, 1983), feline leukemia virus (Jochen, Briddell and Mia, 1983; Lutz, Pedersen and Theilen, 1983; Lutz *et al.*, 1983), bovine leukemia virus (Portetelle *et al.*, 1984), bovine enteric coronavirus (Crouch, Raybould and Acres, 1984) and rotavirus (Sonza, Breschkin and Holmes, 1983).

Monoclonal antibodies against parasites have been amply documented: some examples are *Trypanosoma cruzi* (Araujo *et al.*, 1982), *Trichonella spiralis* (Gamble and Graham, 1983), *Babesia bovis* (Wright *et al.*, 1983) and *Dirofilaria immitis* (Scott, 1983). This area has been reviewed recently by Gamble (1984). Various bacteria have also been studied intensively with monoclonal antibodies. In addition to studies on *Escherichia coli* (Holley, Allen and Barnett, 1984; Mills and Tietze, 1984), monoclonal antibodies have found considerable application in detection of bacteria that are difficult to culture or have very long incubation periods *in vitro*, such as *Mycobacterium* spp. (Kolk *et al.*, 1984; Miller and Buchanan, 1984; Minden *et al.*, 1984; Morris and Ivanyi, 1985; Morris, Torns and Woolley, 1985; Young *et al.*, 1985) and *Brucella* spp. (Holman *et al.*, 1983; Bundle *et al.*, 1984; Schurig, Hammerberg and Finkler, 1984; Bundesen *et al.*, 1985; Sutherland, 1985). Another exceedingly valuable application of monoclonal antibodies is in the rapid diagnosis of life-threatening infections such as *Streptococcus* group B in the central nervous system of the young human (Rensch, Metzger and Baker, 1984) and *Vibrio cholerae* infection (Gustafsson, Rosen and Holme, 1982). Monoclonal antibodies against these and other bacteria and their applications have recently been reviewed by Macario and Macario (1985).

While many types of assay for antigen detection have been described, four are commonly used with monoclonal antibodies:

1. Probably the simplest technique is to attach monoclonal antibody chemically to particles, for example, latex or erythrocytes, which then detect the presence of antigen in fluids by visible agglutination, usually in minutes. A disadvantage of this technique is the inability of small (monovalent) antigen to cause agglutination. In fact, small antigen molecules may block agglutination by larger molecules, thus giving a false negative result.
2. Direct demonstration of antigen in tissue sections, cultured cells or smears by the use of a monoclonal antibody conjugated to a fluorochrome, enzyme (used in conjunction with a substrate that has an insoluble cleavage product) or radioisotope as a detecting agent.
3. A competitive-binding type of assay in which monoclonal antibody is attached to a solid matrix such as the well of a polystyrene microtitre plate. Antigen (not necessarily purified) but conjugated with fluorochrome for enzyme or radioisotope is added only in sufficient quantity to bind all the

available antigen-binding sites of the antibody when premixed with the unknown sample to be tested for presence of antigen. Decrease in binding of the known antigen reflects the presence and quantity of antigenic material in the unknown sample.

4. Antibody immobilized in polystyrene wells can 'trap' antigen in fluid preparations. The trapped antigen may then be detected using another antibody to the antigen. This second antibody may be conjugated with a fluorescent, radioactive or enzymic agent to assist detection. Alternatively, layers of anti-antibody can be added to increase sensitivity or an amplified system may be used (Butler, 1981). For this type of assay, monoclonal antibodies may be used for both the trapping and detection but they must have different specificities for the test to function properly unless the antigen is a single repeating unit such as the O-chain of some bacteria. The most functional test of this type uses a polyclonal trapping antibody and a monoclonal detection antibody which then imparts the required specificity. A schematic representation of these assay types is presented in *Figure 2*.

Although monoclonal antibodies are very useful in the detection of antigens or their components, caution should be exercised in that some monoclonal antibodies may be too specific for general diagnostic use. Van Zaane (1983) reported that two monoclonal antibodies were capable of distinguishing some strains of hog cholera virus from bovine viral diarrhoea virus; however, both antibody preparations failed to react with some strains of hog cholera virus by an immunofluorescence test. It is therefore essential to ascertain the reactivity of the monoclonal antibody with an antigenic determinant present on all antigens to be tested for, before diagnostic use. On the other hand, the exquisite specificity of monoclonal antibodies allows the differentiation of antigens previously thought to be indistinguishable. For example, using a panel of monoclonal antibodies it is possible to differentiate various street strains (i.e. non-laboratory strains isolated from natural sources) and two vaccine strains of rabies virus by comparing immunofluorescence patterns as depicted in *Table 1* (modified from Webster, Casey and Charlton, 1986). Similarly, monoclonal antibody to Marek's disease tumour-associated antigen can be used to identify Marek's disease in chickens. As this antibody does not cross-react with antigens expressed by lymphoid leukosis virus, a differential diagnosis is possible (Lee, Liu and Witter, 1983).

Monoclonal antibodies against a virus have also been used to determine the presence of serum antibody. Anderson (1984) described an enzyme immunoassay in which a blocking monoclonal antibody was used to detect group-specific antibodies against bluetongue virus. Low-affinity monoclonal antibody, reacted with bluetongue virus antigen, could be displaced on the antigen by the addition of ovine or bovine serum containing antibody. Evidently this assay was capable of detecting antibody against all 22 serotypes of bluetongue virus but not antibody against epizootic haemorrhagic disease of deer, caused by a cross-reacting virus. This type of assay has another

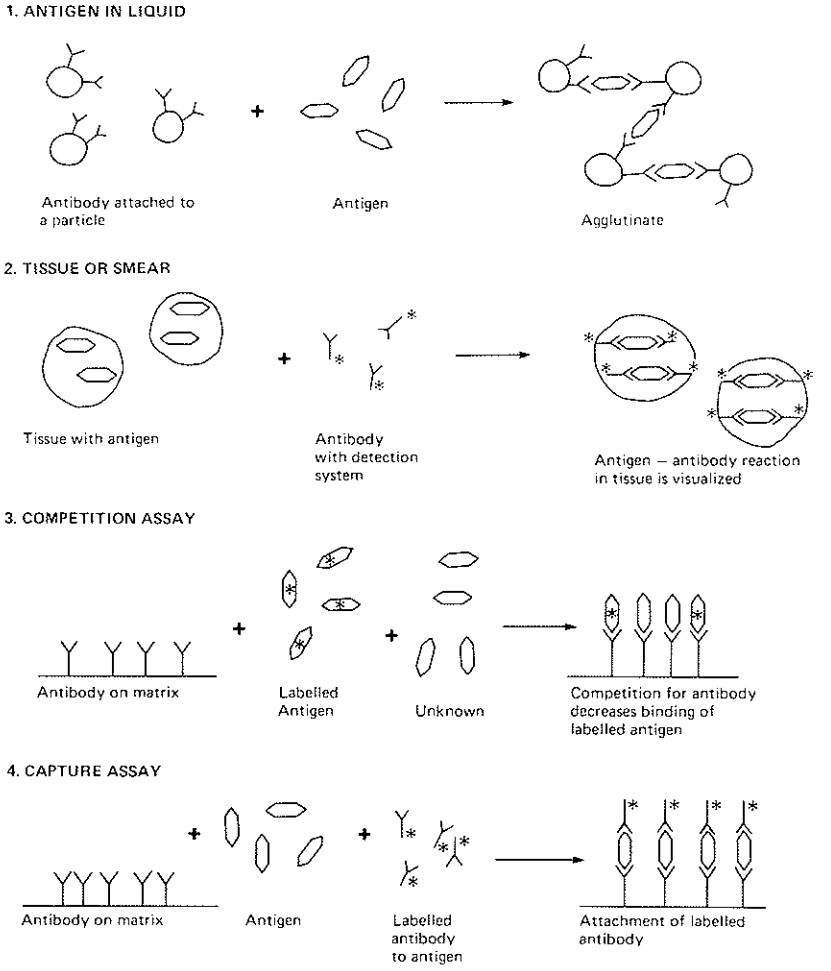

Figure 2. Common techniques using monoclonal antibody for detection of antigen. Briefly, in 1, monoclonal antibody is attached to particles and antigen agglutinates particles. In 2, antigen in tissues or smears is detected directly with monoclonal antibody conjugated with a detection system (fluorochrome, enzyme or isotope). Assay 3, uses monoclonal antibody attached to a solid matrix (for example, a polystyrene well of a microtitre plate) and a known amount of conjugated antigen competes for antigen binding sites on the antibody with antigen present in an unknown sample. In 4, monoclonal antibody attached to a matrix captures antigen which is then measured by a second antibody to the same antigen but conjugated with a detection system. A fuller explanation appears in the text.

major advantage in that no anti-globulin reagent is required and it can therefore be used to detect antibody in serum of any species.

Accurate and rapid diagnosis of infectious agents is of considerable importance in veterinary medicine, particularly when dealing with highly infectious agents such as foot-and-mouth disease virus. Monoclonal antibodies have an obvious role in the diagnostic laboratory; however, if the antibody is not

Table 1. Immunofluorescent reaction patterns of various selected monoclonal anti-nucleocapsid antibodies (denoted W or S) with various Canadian isolates of rabies virus (1 to 10). A + indicates a positive reaction, − is negative and +/− is a variable reaction perhaps indicative of multiple serotypes (data supplied by A. Webster, ADRI)

Monoclonal anti-nucleocapsid antibodies	Antigenic groups*									
	1	2	3	4	5	6	7	8	9	10
W502-2	+	+	+	+	+	+	+	+	+	+
W104-4	+	+	+	+	−	+	+	+	+	−
W239-10	+	+	+	+	+/−	−	+	+	+	−
W102-27	−	−	+	+	−	−	−	−	+	+
W714-3	−	−	−	−	−	−	+	−	−	−
W422-5	−	−	−	−	−	−	−	−	−	−
W701-9	−	+	+	+	−	−	−	−	+	+
S3-1	−	−	+	+	+/−	−	−	+	+	+
S24-1	+	+	+	−	+	+	+	+	+	+
S62-4	+	+	+	+	+	−	−	+	+	+
S97-3	+/−	+/−	+	+	+/−	−	−	+	+	+
S97-11	+/−	+/−	+	+	+	+	+	+	+	+
S143-1	−	−	−	−	−	−	−	−	+	−
S146-3	−	−	+	+	−	−	−	−	+	+

*Antigenic groups as follows: (1) Canadian Arctic (all species of animals); (2) South-east Georgian Bay (all species of animals); (3) South mid-central Canada (skunk); (4) Brooks, Alberta (skunk); (5)–(8) Bat rabies virus; (9) CVS (stock strain of fixed virus); (10) Endurall (vaccine strain)

carefully selected, erroneous diagnoses may result, or results may be obtained that are less satisfactory than those obtained by standard antigen-detection procedures (Phillips *et al.*, 1982). Some criteria for monoclonal antibody selection for diagnostic purposes for antigen detection have been described by Wands *et al.* (1981):

1. The antibody must have sufficient affinity for the antigen to permit efficient combination with low antigen concentrations and to displace efficiently host antibody already attached to the antigen;
2. The antibody should ideally be directed against an epitope of the antigen not recognized by host antibodies and therefore not masked by them;
3. The antibody should be directed against a repeating epitope which is readily accessible, such as occur on lipopolysaccharides of Gram-negative bacteria or virus coat proteins;
4. Multivalency of the antibody may increase test sensitivity. For example, IgM may be more effective than IgG.

In addition to these considerations, the antibody should be stable, should accept conjugation procedures without loss of antigen-binding capacity and should be of the appropriate specificity for its purpose. For instance, in our laboratory several monoclonal antibodies to *Mycobacterium paratuberculosis*

have been produced. Three different clone products were highly specific for the immunizing isolate of this species but did not react with any other isolate of the same species or with any other species of *Mycobacterium* spp. *in vitro*. Part of this problem may reside in the specificity of the monoclonal antibody and/or with the steric presentation of the antigen molecule on the solid phase of an *in vitro* testing procedure. Care should also be taken that the monoclonal antibody is not directed against a host-cell component or contaminant, such as is often present in virus prepared from tissue culture.

IMMUNODIAGNOSTIC REAGENTS FOR ANTIBODY DETECTION

The second use of monoclonal antibody in disease diagnosis is in the detection of antibody most commonly as an anti-globulin reagent conjugated with a detection system (fluorochrome, enzyme or isotope) in a primary binding assay. A variety of different assay types have been devised, some of which are depicted in *Figure 3*; however, the most commonly used is an indirect assay in which the antigen is passively adsorbed to a plastic (polystyrene) surface by hydrophobic interaction. The sample—serum or other body fluid suitably diluted—is applied to the immobilized antigen. After an incubation period, the monoclonal anti-species antibody conjugate is added. Each step is followed by a thorough washing cycle. These are essential steps in which measures are usually taken to eliminate non-specifically bound proteins to reduce 'background' activity in negative samples. The final step if an enzyme conjugate is used is to add a chromogenic substrate followed by spectroscopy, while radioisotope or fluorochrome conjugate binding may be assessed directly. Numerous applications of this type of assay have been reported in the literature as the assay lends itself to most antigens except, perhaps, pure carbohydrates, which may not attach well to polystyrene. For the purposes of this discussion, however, we will deal mainly with data generated in our own laboratory.

In veterinary medicine, diagnostic samples frequently exceed 100 000 per year (for example, in Canada in 1984, 1·6 million samples were tested for antibody to *Brucella abortus*). These numbers eliminate the use of radioisotopes as detecting agents because of the impractical aspects of disposal and handling and as a result most work has focused on enzyme-conjugated monoclonal antibody. Several indirect assays have been developed as diagnostic test procedures: examples of these are the use of lipoarabinomannan from *Mycobacterium paratuberculosis* and the use of smooth lipopolysaccharide from *B. abortus* as antigens to detect antibody in bovine sera to the respective micro-organisms.

In examining bovine sera with antibody to *B. abortus* resulting from vaccination of 7–8-month-old calves with 3×10^9 live strain 19 organisms, using monoclonal antibodies specific for IgM, IgG_1, IgG_2 or IgA in an enzyme immunoassay, it became clear that all four isotypes appeared nearly simultaneously (day 4–5 post-vaccination) although the kinetics of their production differ (*Figure 4*). The specificity of the monoclonal antibodies was ascertained by their reactivity with purified immunoglobulin isotypes

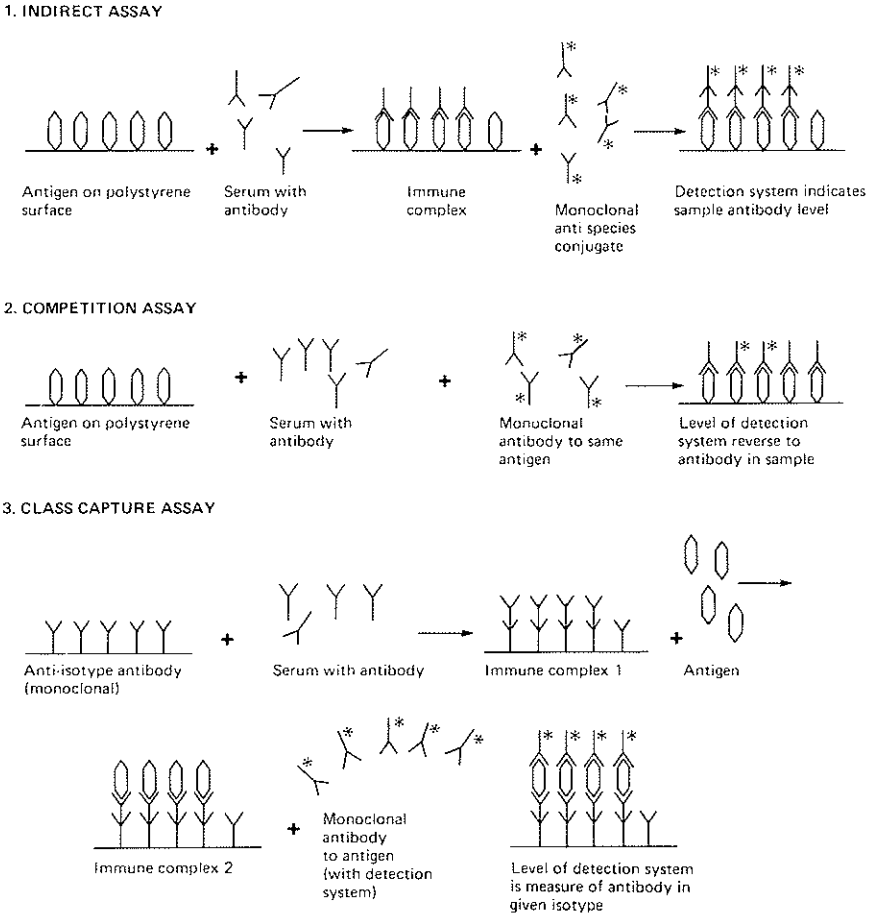

Figure 3. Some types of primary binding assays using monoclonal antibody for detection of serum (or other body fluid) antibody. In the indirect assay (1), antigen is immobilized on plastic (polystyrene), the unknown sample is added and any resulting immune complexes are detected by using a monoclonal anti-species antibody conjugated with fluorochrome, enzyme or isotope. The competition assay (2) also uses antigen attached to a plastic surface. A known amount of monoclonal antibodies to the antigen conjugated with a detection system compete with antibody in a sample for antigenic determinants. Unlike examples 1 and 3, the less monoclonal antibody conjugate that is bound, the more antibody there is in the unknown sample in the competition assay. Class capture assays (3) are useful for detection of IgM and IgG antibodies to an infectious agent. Presence of IgM antibody indicates recent exposure but is frequently difficult to measure because of the larger amounts and higher affinity of IgG antibody. Briefly, a monoclonal antibody against a given isotype, for example IgM, is immobilized on a matrix. The test sample is added and, presumably, with an even distribution of immunoglobulin of all specificities being captured, some antibody of that isotype to the antigen will be captured as well. This antibody will react with the antigen and the whole reaction may be assessed by addition of monoclonal antibody to the antigen to which a detection system has been conjugated.

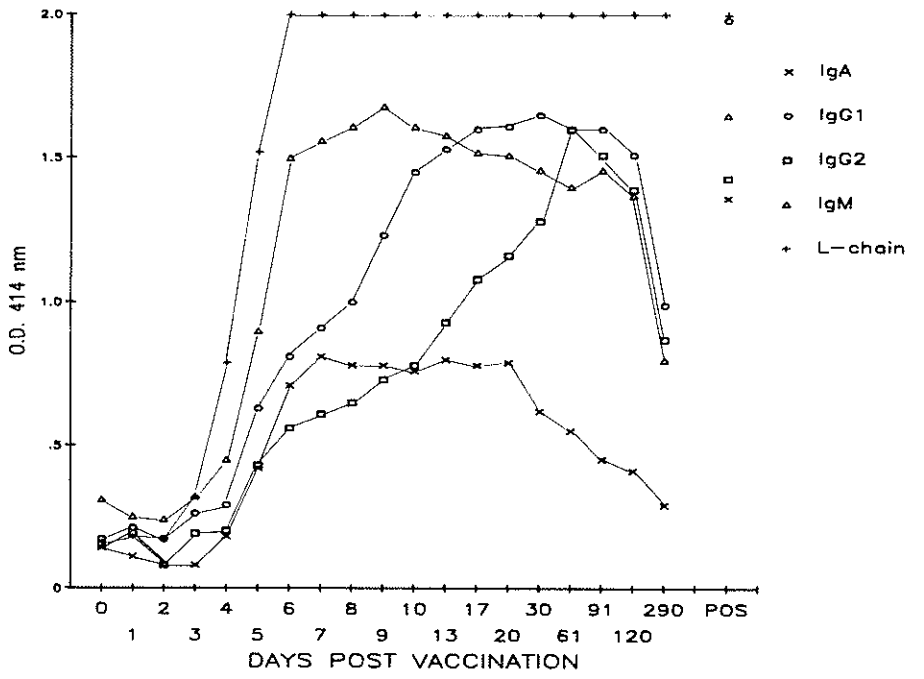

Figure 4. Enzyme immunoassay results plotted against time after vaccination with 3×10^9 *B. abortus* strain 19. Graphs represent the average data of six animals using serum dilutions of 1:100 and *B. abortus* S-LPS as the antigen. Monoclonal antibody to IgA, IgG_1, IgG_2, IgM and L-chain were conjugated with horseradish peroxidase and used at 80 ng of antibody per test. POS = positive (field infected). For the serum from field-infected cattle, results obtained with five sera of intermediate titres were averaged. Note that, for the field-infected sera, IgM and IgA determinations were performed at a dilution of 1:200 while IgG_1, IgG_2 and total antibody (L-chain) determinations were done at a 1:100000 dilution.

attached directly or as immune complexes to polystyrene plates. These data have been confirmed several times, including an experiment using adult cattle. It is of particular interest that IgM antibody production persists at high levels with simultaneous production of the IgG subclasses: the continuous production of IgM thus contributes to the persistent 2-mercaptoethanol-sensitive agglutination titres in vaccinates. IgA antibody is readily detectable in post-vaccination serum for several months. Similarly, in quantitating antibody isotypes of sera from cattle infected in the field with *B. abortus* or experimentally infected with strain 2308, it was found that elevated IgM antibody levels (of diagnostic importance) persist in sera of cattle with long-standing infection.

Some of these data are presented in *Figure 4*. The literature contains several reports of IgM isotype of antibody predominating in the early stages of infection (Rose and Amerault, 1964; Rose and Roepke, 1964; Rose, Lambert and Roepke, 1964; Beh, 1974) while in other cases IgG appear to

be prevalent (Rice, Tailyour and Cochrane, 1966; Beh, 1974) in serum. These data presumably depended on the type of test used for their determination.

In addition, in all sera examined from cattle infected with *M. paratuberculosis* that contained antibody, substantial amounts of the IgM class antibody were found as well as the other isotypes. On the basis of these data, IgM may play an important part in serological diagnosis and it was decided that a monoclonal antibody of choice would react with the bovine immunoglobulin light (L) chain, thus resulting in a global estimate of antibody activity in a serum, albeit biased toward IgM detection. Of several anti-L chain clones propagated, all but two were found to be non-reactive with immunoglobulins of all classes from approximately 3% of the cattle population. The exact specificity of these monoclonal antibodies is not yet known; however, they are obviously unsuitable as diagnostic reagents. In fact, once again it is clear that the monoclonal antibodies must be extensively tested for specificity before use in the diagnostic laboratory to avoid false results. Of the remaining two clones, one with a titre of over 1:100 000 was selected, tested against some 700 bovine sera and found to react in all instances. Ascites-fluid-derived antibody from this clone was labelled with horseradish peroxidase (HRPO, EC 1.11.1.7) and tested extensively to establish the quality control parameters essential to a continuously performed enzyme immunoassay. The variability in substrate development with a single serum (with antibody to *B. abortus*) repeated in duplicate on 25 different 96-well plastic plates is shown in *Figure 5a*. These data indicate an acceptable performance of the assay in that all 50 determinations fall within two standard deviations (80% fall within one standard deviation). As seen in *Figure 5b*, occasional 'outliers' of results occur, prompting rejection of that particular assay.

In our view, an enzyme immunoassay performed in a 96-well plate for diagnostic serology should contain three controls, with a fourth being optional. Thus a 96-well plate is divided into quadrants as previously described (Nielsen and Wright, 1984): a positive control, diluted to result in the development of an optical density of 1·0 in approximately 10 minutes (using the targeting protocol of Wright, Kelly and Gall, 1985), is placed in wells A1 and E7; a positive serum of low reactivity (optical density of about 0·5) is placed in wells B1 and F7, while wells C1 and G7 contain wash buffer in place of serum. If any or all of these controls result in optical densities outside the two standard deviations calculated by repeating these samples several hundred times, that particular test is rejected and repeated. The optional control is a highly positive sample to ascertain maximum optical density development.

In *Figure 6*, antibody results of samples from cattle at various times after vaccination with *B. abortus* strain 19, determined with the monoclonal anti-L chain (based on reactivity with the four major immunoglobulin isotypes and isolated L-chain) or a polyclonal anti-IgG (H+L chain) detecting antibodies are presented. It is quite evident that the anti-L chain conjugate favours detection of IgM antibody in 'early vaccinates', whereas lower activity compared with the polyclonal conjugate is observed in 'late vaccinates'.

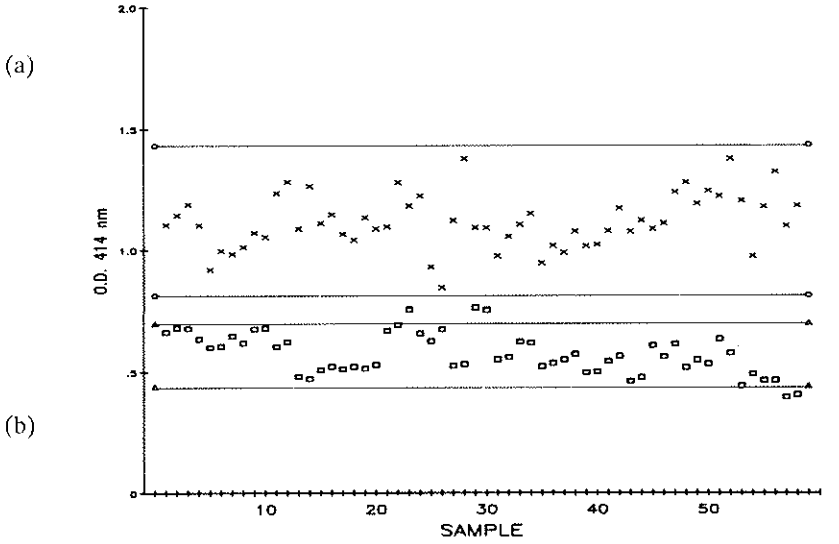

Figure 5. Quality control charts (a) using a single serum with antibody to *B. abortus* diluted 1:10000 (××××) and (b) using a single serum with antibody to *B. abortus* diluted 1:100 (□□□□). In both cases *B. abortus* S-LPS was used as antigen and a mouse anti-bovine L-chain monoclonal antibody conjugated with horseradish peroxidase as a detection reagent. Each sample was determined in duplicate on 25 different 96-well plates over several days. Note that the high-titre serum determinations all fall within two standard deviations (indicated as ○–○ for the high-titre and △–△ for the low-titre serum) while with the low-titre serum several 'outliers' are encountered.

Overall, the diagnostic sensitivities of the monoclonal and polyclonal reagents appear to be similar, while the specificity of the monoclonal conjugate may be enhanced by its failure to detect 'late vaccination' antibody. The monoclonal antibody has the advantage of being derived from an 'immortal' source and its enzyme conjugate appears to be stable for at least 6 months (stored at 4°C filter-sterilized). Enzyme conjugation of seven individual batches has proved to be uniform, based on performance and the number of haem groups of the enzyme compared with the amount of antibody. The cost of the monoclonal antibody–enzyme conjugate based on testing 250 000 samples is approximately one-third that of the polyclonal antiglobulin conjugate. Rat monoclonal antibodies currently are being produced and evaluated. The rat monoclonals are less expensive to produce and are more stable than their murine counterparts (Galfré, Milstein and Wright, 1979).

Primary binding assays are currently employed only to a small extent in veterinary medicine. However, with suitable apparatus becoming more commonplace, primary binding assays and, in particular, enzyme immunoassay will replace current serological tests (complement fixation test, serum neutralization test, precipitin test, agglutination test). In order to compare data obtained in one laboratory with that of another, or even from the same laboratory over the years, standardized reagents are essential. Polyclonal sera can be prepared and conjugated with enzyme in large batches; however,

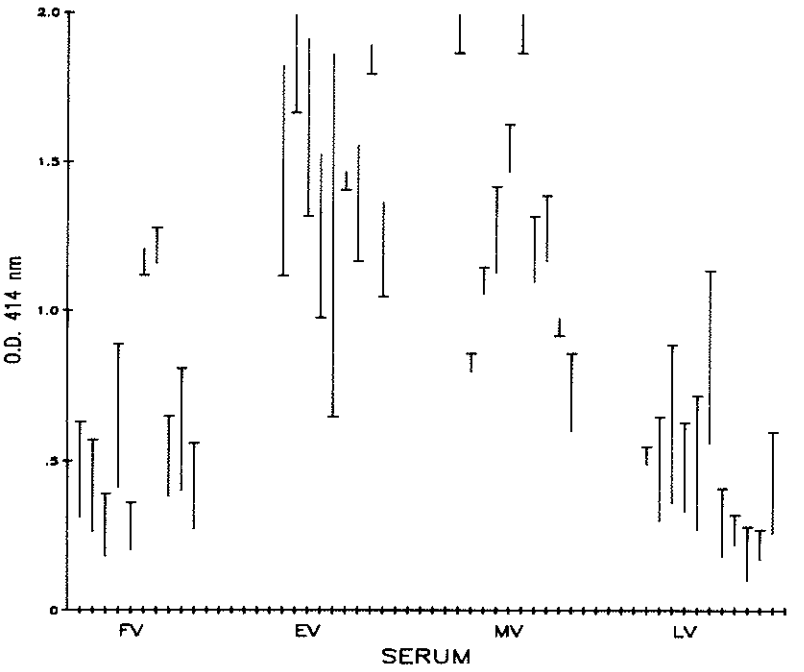

Figure 6. Antibody determinations using an indirect enzyme-immunoassay in sera from cattle vaccinated or infected with *B. abortus*. *B. abortus* S-LPS was used as the antigen, all serum dilutions were 1:100 and a monoclonal anti-bovine L-chain antibody and a rabbit anti-bovine IgG (H- and L-chain) antibody both conjugated with horseradish peroxidase were used as detecting agents. Each point represents a duplicate determination for a single serum, the horizontal line being the result with the polyclonal antibody conjugate and the vertical line indicating the higher or lower result with the monoclonal antibody conjugate. Sera from early vaccinates (EV, up to 120 days after vaccination) give higher results with the monoclonal antibody conjugate, presumably because of the substantial amount of IgM class antibody. Sera from later after vaccination (LV, over 300 days) give lower results with the monoclonal antibody conjugate than with the polyclonal antibody conjugate, probably because of the predominance of IgG_1 classes of antibody in the sera. MV refers to sera from cattle vaccinated 120–300 days previously, while FV are sera from adult animals vaccinated as calves and obtained at random. No trends are noticeable with sera from field-infected cattle.

they will always differ from lot to lot with respect to their specificity and ability to conjugate. Monoclonal antibody, on the other hand, theoretically is inexhaustible and thus antibody of identical specificity can be prepared at will and the enzyme-conjugation procedure results in no discernable differences from batch to batch. Thus a cell-line product of suitable specificity should be made available to all interested parties, together with a detailed protocol for its handling, use and quality control steps to facilitate national and international standardization of primary binding assays.

IMMUNODIAGNOSTIC REAGENTS: ANTI-IDIOTYPE ANTIBODY

A somewhat different approach to diagnostic serology is the use of anti-idiotype antibody to detect antibody with a given specificity (*Figure 7*). Anti-

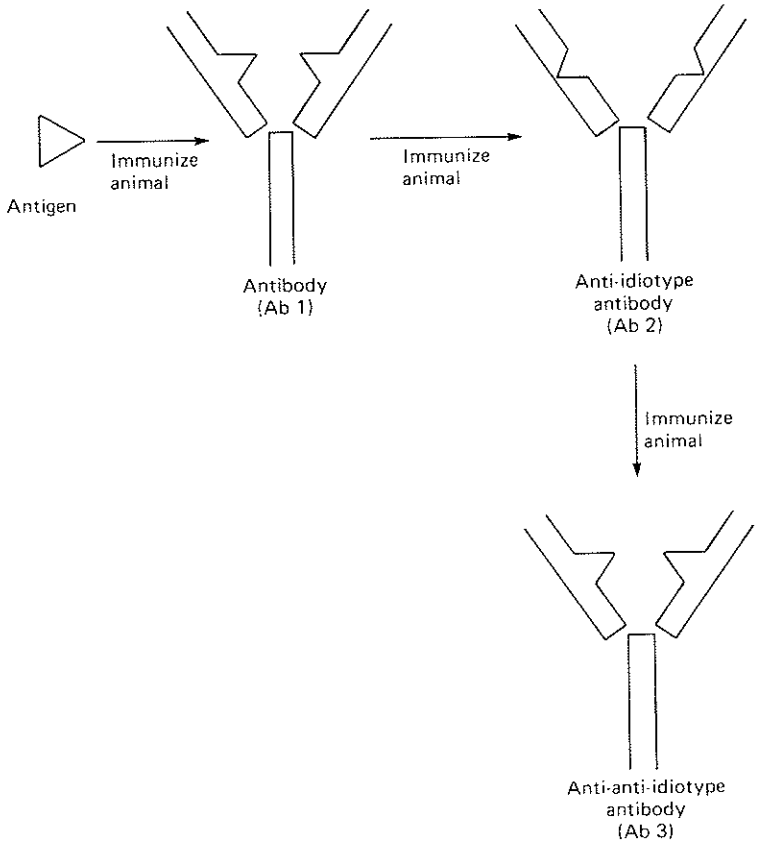

Figure 7. An animal immunized with the antigen produces antibody (Ab 1). If Ab 1 in turn is used as an antigen in a second animal, anti-idiotype antibody (Ab 2) will result. Using Ab 2 as an antigen in a third animal results in the production of anti-anti-idiotype antibody (Ab 3) which has the same specificity as Ab 1.

idiotype antibody (Ab 2) has, by definition, antibody activity to the antigen-combining amino acid epitope sequence (of the folded molecule) of the hypervariable region of the antibody (Ab 1) molecule. Anti-idiotype antibody can be prepared by immunization of a heterologous species or a homologous species (without Ab 1) with affinity purified Ab 1. The resulting Ab 2 should be able to compete with the original antigen for the antigen-binding sites of Ab 1. If a polyclonal Ab 2 is made, even in a homologous species, it generally has to be absorbed extensively to remove anti-species or anti-allotype activity. If Ab 2 is monoclonal, it (as well as a polyclonal Ab 2) would have to be tested exhaustively with immunoglobulins with or without Ab 1 activity and with or without the F_c portion to eliminate non-specific reactions that often occur between immunoglobulins and, for example, bacteria such as *Staphylococcus* spp. and *Streptococcus* spp. The monoclonal Ab 2 can be used in a primary binding assay as a capture agent (*see Figure 7*) for Ab 1. The Ab 1 bound may then be detected with a second monoclonal antibody with specificity for a different portion of Ab 1 and conjugated with a

detection system such as an enzyme. If Ab 2 was raised in a homologous species, Fab of Ab 2 should be used for capture while an anti-F_c reagent-conjugate should be used for detection. An alternative approach is to attach Ab 2 chemically to particles such as erythrocytes or latex beads and passive agglutination should take place in the presence of Ab 1. By either method, detection of antibody may be accomplished in the absence of antigen, a technique which may prove useful for serology that otherwise involves antigens that may be very expensive, toxic or difficult to obtain. Anti-idiotype antibody has been made against antibodies with specificity for phosphorylcholine (McNamara, Ward and Köhler, 1984), levan (Bona *et al.*, 1984); *E. coli* (Stein and Sonderstrom, 1984), various bovine pathogens including *Staphylococcus* spp. and *Streptococcus* spp. (Arulanadam, Sevoian and Goldsby, 1985) and to *B. abortus* (K.H. Nielsen, M.D. Henning and J.R. Duncan, unpublished work).

Experimental uses of monoclonal antibodies

CELLS OF THE IMMUNE SYSTEM

Monoclonal antibodies have been used extensively to characterize the various cell populations involved in immune responses of mice and man. Thus the Lyt system of antigens has been developed for functional differentiation of T-lymphocytes and in a similar vein the Lyb antigens for B-lymphocytes. Similar studies in domestic animals are in their infancy and only a few are in evidence in the literature. Monoclonal antibodies have been used to characterize carp lymphocytes (Fiebig *et al.*, 1983) and for analysis of lymphocyte heterogeneity in the same species (Egberts *et al.*, 1983). Krawiec and Muscoplat (1984a,b) developed monoclonal antibodies specific for canine T-lymphocytes and others capable of distinguishing medullary and cortical thymocytes. Equine lymphocyte surface antigens have been studied by Newman, Beegle and Antczak (1984) as well as IgM on the lymphocyte surface (and in serum) of immunodeficient and healthy horses (McGuire, Perryman and Davis, 1983) and in pregnancy (Antczak, 1984). The cells of the bovine immune system have received more attention, starting with the work of Pinder, Pearson and Roelants (1980) and of Pinder *et al.* (1980) who developed monoclonal antibodies to lymphocytes as well as to IgM, both on the cell surface and in serum. Subsequently, Lewin *et al.* (1984a) and Lewin, Davis and Bernoco (1985) produced three monoclonal antibodies that distinguished T and B lymphocytes of cattle in peripheral blood and used these monoclonals in a study of a bovine leukemia-infected herd to show that no consistent differences in B-cell number resulted from the virus infection (Lewin *et al.*, 1984b). Similarly, Rabinovsky and Yang (1984) produced reagents that were capable of reacting with T-lymphocyte subsets based on immunofluorescence patterns of cells from lymph nodes and peripheral blood. In a very extensive study, Davis, Perryman and McGuire (1984) catalogued about 100 monoclonal antibodies for their reactivity patterns with bovine, ovine, caprine, porcine, equine and human lymphocytes. Reactivity of the monoclonal antibodies based on immunofluorescence analysed by a

fluorescent antibody cell sorter resulted in patterns suggesting specificity with various T-lymphocyte subsets, including suppressor/cytotoxic cells, as well as pan T-lymphocyte reagents and both T and B cells; class I and class II MHC antigens; monocytes, granulocytes and erythrocytes, and IgM (B-cells) and light chains. Interestingly, interspecies cross-reaction was extensive with some reagents and limited with others, with monospecificity being relatively rare to some antigens, such as MHC-class I antigen. In addition, Spooner and Pinder (1983) have reported on monoclonal antibodies evidently detecting MHC antigens while Letteson *et al.* (1983) could detect 'Ia-like' antigen also in cattle. Finally, Lunney (1984) used monoclonal antibody to swine lymphocyte antigen complex to identify and purify Ia antigens and to study the immune response to various antigens including a synthetic peptide, myoglobin and lysozyme in high and low responder swine. Lunney, Pescovitz and Sachs (1984) also produced reagents to segregate peripheral blood lymphocytes of swine into B-lymphocyte populations, T-lymphocytes and macrophages and subpopulations of T-lymphocytes.

It is obvious that the tools for analysis of the immune response in animals other than mouse and man are being developed and, eventually, exact definition of involved cells will be possible. This is a very important area of research that will, we hope, lead to a selection of animals genetically resistant to disease.

PREGNANCY AND SEX DETERMINATION

Booman *et al.* (1984) have described the application of monoclonal antibodies to detect pregnancy by the presence of progesterone in milk. Using a direct double-antibody solid phase enzyme immunoassay they found test sensitivity to be 1–2 pg per ml of progesterone, which was excellent for a 5-hour assay completed without extraction or centrifugation of the milk.

Monoclonal antibodies have been prepared to a variety of other hormones including somatotropin (Krivi and Rowold, 1984), human chorionic gonadotropin, and bovine and porcine insulin (Schönherr, Roelofs and Houwink, 1984) and to zeranol (Carter, Dixon and Bew, 1984).

An interesting and, at present, controversial application is in the area of embryo sexing. Antibody to H-Y antigen, the male specific transplantation antigen, has been sought over the years to establish definitively the sex of an embryo. In general, polyclonal antisera were unsatisfactory because of their inconsistencies in detecting H-Y antigen and their low titres after absorption. Monoclonal antibodies, on the other hand, have not turned out to be the expected solution to the problem (Nagamine, Reidy and Koo, 1984). Koo *et al.* (1981) reported on monoclonal antibodies that were cytotoxic to lymphoid cells, while Hall and Wachtel (1980) produced monoclonal antibody capable of precipitating H-Y antigen excreted by Sertoli cells; however, the latter findings could not be confirmed by Gore-Langton, Tung and Fritz (1983). This led Zenzes and Reed (1984) to suggest that perhaps H-Y antigens cannot be identified by serological methods, whereas Warner *et al.* (1985) suggested that they can. Whatever the truth is, the determination

of the sex of an embryo without damaging the cells would be of immediate use in the animal industry, especially with cattle in which embryo transfer is commonplace but sexing is done by karyotyping at present.

MOLECULAR STRUCTURE OF ANTIGENS

Classically, antigens are defined as the substance capable of reacting with antibodies; however, the reactivity with antibody is confined to antigenic determinants or epitopes present on the antigen molecule. The epitope complements the antigen-binding site of the variable region of the antibody molecule in its configuration. It is generally accepted that the invagination formed by the hypervariable regions of the antibody molecule is similar in size to a peptide of six to eight amino acids in length. Monoclonal antibodies have greatly facilitated these studies because of their homogeneity with respect to affinity, size, class and fit on the antigen. The valency or number of epitopes of an antigen can be approximated by determining the number of antibody molecules that bind simultaneously. This is, however, usually a low estimate of the number of epitopes because of steric hindrance of antibody molecules. Epitopes of antigens fall into two broad categories of 'sequential' and 'conformational' determinants (Sela, 1969): as their names imply, sequential determinants are sequences of amino acids of an unfolded or randomly folded peptide, whereas conformational epitopes are the amino acids brought into close proximity by the conformation of the antigen; as a consequence, antibody to a true conformational determinant rarely reacts with the unfolded molecule. A conformational determinant is a rather arbitrary antigen because, presumably, an unfolded molecule could have the same sequence and therefore react with antibody. Atassi and Smith (1978) suggested that 'continuous' and 'discontinuous' determinants would be more suitable names. A continuous determinant is, as the name implies, a continuous sequence of residues on the surface of an antigen possessing conformational folding. Thus, discontinuous determinants can become continuous determinants by molecular folding. Other epitopes, such as topographic determinants, cryptotopes and neotopes, have been described and recently reviewed in detail by van Regenmortel (1984). The use of synthetic peptides to mimic antigenic regions of proteins has led to successful delineation of epitopes of several antigens, for example myoglobin (Reichlin and Noble, 1977). Similarly, enzymatic cleavage of proteins has been used as a tool to study epitopes (Atassi, 1975).

A considerable number of protein antigens and viruses have been studied in detail with monoclonal antibodies to determine their epitope structures, including lysozyme (EC 3.2.1.17) (Metzger, Miller and Sercarz, 1980; Kobayashi *et al.*, 1982; Smith-Gill *et al.*, 1982) and the haemagglutinin antigen of influenza virus (Koprowski, Gerhard and Croce, 1977; Yewdell and Gerhard, 1981).

The molecular dissection approach has been used with monoclonal antibodies to select antigenic variants of several viruses of veterinary importance. Wiktor and Koprowski (1980) and Flamand, Wiktor and Koprowski (1980)

reported selection of naturally arising antigenic variants based on the glycoprotein antigen of rabies virus. Using three monoclonal antibodies, Coulon *et al.* (1982a) selected variants and reported that one of the antibodies was capable of selecting avirulent viruses similar to the parent virus except in their capacity to induce an immune response (Coulon *et al.*, 1982b). Using a more extensive monoclonal antibody panel, Coulon, Rollin and Flamand (1983) developed a map of antigens (epitopes) related to virulence. A single amino-acid change—arginine at position 333 of the glycoprotein in the virulent form of the virus—was found to be the only change in non-pathogenic variants (Dietzschold *et al.*, 1983). Functional protein antigens have been demonstrated on other micro-organisms, using monoclonal antibodies. Neutralizing antibodies to antigens such as the VP 2 antigen in bluetongue virus (Appleton and Letchworth, 1983; Letchworth and Appleton, 1983a,b), the gp 56 antigen of Venezuelan equine encephalitis virus (Roehrig, Day and Kinney, 1982), foot-and-mouth disease virus VP 1 antigen and its peptides (Meloen *et al.*, 1983; Robertson, Morgan and Moore, 1984), to bacterial antigens, for example streptococcal M protein and its peptides (Hasty *et al.*, 1982) and *B. abortus* lipopolysaccharide (Montaraz *et al.*, 1986) and to parasite antigens such as *Plasmodium berghei* (Yoshida *et al.*, 1980), *Plasmodium knowlesi* (Thomas *et al.*, 1984) and *Leishmania* (Anderson, David and McMahon-Pratt, 1983), have been described.

The list of micro-organisms is not meant to be exhaustive and only a few well-established findings have been referenced. It is intended to demonstrate how monoclonal antibodies applied initially to basic research problems can eventually lead to technology applicable to applied areas such as veterinary diagnostics and subunit vaccine production by the elucidation of diagnostic and protective antigens. These findings in turn will lead to production of subunits with biological activity which may allow their mass production as diagnostic reagents and elimination of the use of live micro-organisms for vaccine. Such research also leads to other approaches: thus, cross-reaction among serotypes of antigens may be established. This has found particular use with avian viruses, not only for epidemiological purposes but also for characterization of common antigenic determinants, for example, with Marek's disease virus and turkey herpesvirus (Ikuta *et al.*, 1983; 1984a,b), Rous sarcoma virus (Parsons *et al.*, 1984); Newcastle disease virus (Nishikawa *et al.*, 1983; Russell and Alexander, 1983; Russell, 1984), avian influenza virus (Fukushi, Yanagawa and Kida, 1982) and avian infectious bronchitis virus (Snyder and Marquardt, 1984). The reverse, the exquisite specificity of monoclonal antibodies, can also be used to advantage in that it allows for distinction of antigens of closely related micro-organisms, for example, bluetongue virus and epizootic haemorrhagic disease virus (Jochim and Jones, 1983) and street, vaccine and laboratory strains of rabies virus (Smith, Summer and Roumillat, 1984; Whetstone *et al.*, 1984). Epitope studies can also lead to immunization against viruses to block cellular receptors for the virus, either directly (Minor *et al.*, 1984) or as an anti-idiotype antibody (Sharpe *et al.*, 1984). Monoclonal antibodies have been extensively used in the study of tumour-causing viruses and the survival and function of tumour

virus proteins in host cells. Examples include monoclonal antibody against the p15E protein of C type retroviruses. This protein is highly immunosuppressive and is capable of blocking the monocyte chemotactic response which may protect the tumour cell (Cianciolo *et al.*, 1981). Monoclonal antibodies have also been developed against the transformation-specific glycoprotein coded for by feline leukemia oncogene v-fms (Anderson *et al.*, 1982), avian retrovirus reverse transcriptase (Weis *et al.*, 1983), bovine leukemia virus transformed sheep cells (Hamada *et al.*, 1984) and Marek's disease virus tumour-associated antigens (Liu and Lee, 1983).

AFFINITY CHROMATOGRAPHY

Because of their exquisite specificity, monoclonal antibodies are suitable for use in purification of antigens. This usually entails the immobilization of the monoclonal antibody on an insoluble matrix, such as agarose beads, by a covalent linking agent, for example cyanogen bromide. Beads prepared with a linking agent are commercially available, or can be made fairly readily in the laboratory (Johnson and Garvey, 1977) at a fraction of the cost. The beads with monoclonal antibody attached can be used either by a batch procedure or by column chromatography for antigen purification. The batch procedure involves mixing the gel–antibody with the material containing antigen (end-over-end mixing to avoid breakage of the fragile agarose beads). The mixture is then placed in a Buchner funnel, washed extensively with a neutral isotonic buffer and then the antigen is desorbed by acid or chaotropic dissociation of the antigen–antibody complex. This procedure is advantageous in its rapidity, which also prolongs the life of the antibody attached to the beads; it requires very little equipment and it lends itself to large volumes. Disadvantages include its labour intensiveness and the constant need of attention to keep the gel moist. Affinity chromatography performed in columns makes use of the same generation and dissociation principles of the immune complexes formed. In a laboratory equipped for chromatography, such columns can run with very little time investment on a continuous basis. Disadvantages include the column size, which usually is kept fairly small to avoid prolonged exposure of the antibody to dissociation conditions, and it is a fairly slow procedure (e.g. 6–8 h) compared with the batch method (2–3 h). Antigen purification by affinity chromatography has been applied to substances ranging from Ia antigens of the swine lymphocyte antigen complex (Lunney, 1984), to glycoproteins of herpes viruses (Eisenberg *et al.*, 1982; Arvin *et al.*, 1983), to antigens of *Trichonella spiralis* (Gamble and Graham, 1983); however, according to the literature, extensive use in veterinary medicine has yet to come. The technique has great potential, especially for purification of antigens from complex mixtures containing related or physicochemically similar substances: thus, subunits to be used for serological purposes and isotypes of immunoglobulins for standardization and immunochemical studies would be prime candidates.

Immunochemical applications of monoclonal antibody

Molecular structure of antigens was discussed in a previous section. These studies have been greatly facilitated by application of monoclonal antibodies to immunoblotting analysis. Digested, degraded or native antigen mixtures are separated by standard procedures such as one-or two-dimensional polyacrylamide gel electrophoresis. Usually more than one replicate is run: one gel is stained directly while others may be subjected to electroblotting, thereby transferring the separated materials on to nitrocellulose (Towbin, Staehelin and Gordon, 1979). The nitrocellulose sheet is then blocked with a non-reactive protein and incubated with the monoclonal antibody of choice. Mouse monoclonal antibody binding to antigen may be detected by the antibody being conjugated with its own detection system, for example, an isotope or enzyme; alternatively, an indirect system using an anti-mouse immunoglobulin conjugated with a detection system as the second layer may be employed. Several methods are available for either purpose; however, the immunoperoxidase technique described by Hawkes, Niday and Gordon (1982) has advantages in that it provides very low background activity, is highly sensitive and eliminates the use of isotopes. This Western blot technique has been widely applied to the study of protein antigens, for example, outer membrane protein III of *Neisseria gonorrhoeae* (Swanson, Mayer and Tan, 1982), the heavy chain of myosin (Clark, Frogner and Zak, 1982), fibronectin (Pierschbacher, Hayman and Rouslahti, 1981), spectrin (Yurchenco *et al.*, 1982) and to specificity determinations of monoclonal antibodies to porcine IgG (Hollingshead, Tonkonogy and de Buysscher, 1985).

Monoclonal antibodies to animal immunoglobulins have been prepared and some of their uses have been described above. Antibody to bovine IgM was reported by Pinder *et al.* (1980) to react with IgM in serum and on the surface of lymphocytes. Antibody has also been prepared to bovine IgG_2 (Srikumaran, Goldsby and Guidry, 1981; Srikumaran, Guidry and Goldsby, 1982; Mathison, Kelly and Davis, 1984); bovine IgG_1 (Fleenor *et al.*, 1984) and to bovine IgG, IgG_1, IgG_2 and IgA (van Zaane and Ijzerman, 1984). Interestingly, in the latter report monoclonal antibody to IgM cross-reacted with IgA, a finding observed with some but not all anti-IgM clone products in our laboratory (K.H. Nielsen, M.D. Henning and J.R. Duncan, unpublished work). In addition, monoclonal antibody to porcine IgM (Paul, van Deusen and Mengeling, 1985) and to canine IgG subtypes has been produced (Fuller and Hurrell, 1985). It is our opinion that for monoclonal anti-species immunoglobulin antibodies to reach their full potential as diagnostic/research reagents, uniformity in specificity must be achieved. The reagents decided upon must then be made available to everyone interested, possibly via a commercial concern. It is important that several steps are taken. First, the specificity should be ascertained by several laboratories. Exhaustive tests must be performed to avoid pitfalls, such as antibody to genetic determinants that are not present in all individuals of a species. This problem was reported by Jefferis and Ling (1984) with anti-human IgG_3 which did not react

with proteins from Caucasians but did with proteins from Mongoloid races. Secondly, the affinity of a monoclonal may vary, depending on the antigen presentation. Thus the type of test in which the monoclonal would be used should also be used for screening and specificity assessment. This phenomenon of 'assay specificity' of monoclonal antibody was assessed by Haaijman *et al.* (1984). Thus for the utilization of monoclonal antibody for serology in veterinary medicine a large comparative study should be done, such as that reported for human IgG subtypes (Jefferis *et al.*, 1985) in which the specificities of 74 clone products were tested. In this paper it was again pointed out that the antibody should be tested for specificity and reactivity in the type of assay in which it will be used and that 'epitope display is influenced by physical and chemical procedures used to immobilize or fix antigen'.

In our laboratory we are currently assaying monoclonal antibody specificity in three ways: first, with the isotypes passively adsorbed on to polystyrene; secondly, with a panel of 500 'normal' sera (also passively adsorbed to polystyrene) and thirdly, with an antigen–antibody complex in which the antigen is immobilized and the antibody presumably is in its native state to allow the monoclonal antibody to react with the F_c end of the molecule exposed. The latter assay invariably yielded the highest 'titres' with the monoclonal antibodies; however, it is unfortunately the most expensive in terms of reagents. The above steps are necessary to utilize monoclonal anti-immunoglobulins to the full in order to standardize procedures. Diagnostic serology procedures can be standardized further by the use of a monoclonal antibody as the primary antibody (i.e. to bind to the antigen). This should be fairly readily accomplished by cross-species fusion as reported for bovine × murine (Srikumaran, Guidry and Goldsby, 1984; Hague *et al.*, 1985) and porcine × murine (Raybould *et al.*, 1984). The cross-species fusions in some cases resulted in production of immunoglobulin with characteristics of the bovine or porcine fusion partner. Although these hybrid cells could not be used for ascites fluid production, substantial amounts of immunoglobulin could be produced in tissue culture. For these bovine or porcine monoclonal immunoglobulins to be of value as reagents to standardize serological tests, they must have antibody activity to antigens. This has been accomplished in the case of some bovine pathogens (Goldsby, Hague and Sevoian, 1985) and this approach could presumably be extended by immunization of the donor animal or its cells *in vitro*. Thus it should be possible to produce a reagent that, when used in an indirect enzyme immunoassay in conjunction with a monoclonal anti-immunoglobulin reagent and a chemically defined antigen, meets quality assurance standards of the test on a day-to-day or laboratory-to-laboratory basis. This should reduce the variation observed in *Figure 5*, thereby improving assay performance.

An alternative approach to bovine (or other species) monoclonal antibody production is transfection. Monoclonal antibody and transfection techniques were recently reviewed by Davis *et al.* (1985). Transfection of DNA from cultured tumour cells into spleen cells results in cells with the appearance of

lymphoblasts that can be cultured continuously and secrete immunoglobulin at the same rate as mouse hybridoma cells (Editorial, 1983). Presumably the spleen cells could be pre-immunized to impart specificity to the antibody. The lack of availability of myeloma cell lines from the large domestic animals may be overcome by this technique and it should be possible to produce ascites fluid in genetically similar or immunosuppressed animals.

In an assay system using a chemically defined antigen, a monoclonal first antibody and a monoclonal antiglobulin reagent conjugated with a detection system, a remaining reagent variable is the conjugation procedure. The chemical linkage of, for example, an enzyme such as horseradish peroxidase to an immunoglobulin molecule may result in a certain amount of denaturation attributable to reducing agents (sodium borohydride) or aggregation attributable to glutaraldehyde, depending on which labelling procedure is used. In addition, minor variations in conjugation efficiency may occur, possibly resulting in small discrepancies in the standardization. These problems may be overcome by the use of bifunctional or heteroligating monoclonal antibody.

Bifunctional antibodies have been produced by Milstein and Cuello (1983, 1984) by fusing two hybridoma cell lines, one producing antibody to peroxidase and one to somatostatin, resulting in a single cell that produced antibody molecules of dual specificity. They found that some of the resulting hybrid hybridoma products were very useful for immunohistochemical staining when applied to sections containing somatostatin in the presence of peroxidase. Other hybrid monoclonals were prepared for simultaneous localization of two antigenic sites (Milstein and Cuello, 1984). Bispecific antibodies have also been produced by chemical reassociation of monovalent Fab fragments of monoclonal antibodies of the same isotype using a dithiol-complexing agent with a thiol-activating agent (Brennan, Davidson and Paulus, 1985). This procedure evidently yielded 50–70% bifunctional antibody, in this case with specificity for β-galactosidase and horseradish peroxidase, or β-galactosidase and avidin, or peroxidase and avidin. A somewhat simpler approach may be the use of enzyme–anti-enzyme monoclonal antibody as a detection system bound to immobilized immune complexes via an anti-mouse immunoglobulin bridging reagent (Ternynck, Gregoire and Avrameas, 1983). It is clear that antibodies with specificity for enzymes may be very useful reagents, either as bifunctional antibodies or as soluble immune complexes for serological purposes.

Monoclonal antibodies have proved useful in several other immunochemical areas. The study of the complement cascade has been enhanced by the use of monoclonal antibody of various isotypes in a haemolytic system. It was shown by Neuberger and Rajewsky (1981) that murine IgG_{2a}, IgG_{2b} and IgG_3 could effectively cause lysis whereas IgD could not. Of five IgG_1 preparations tested, two resulted in weak haemolysis and IgM was highly haemolytic. Activation of the human complement cascade has been investigated by Heinz *et al.* (1984) and Kilchherr, Schumaker and Curtiss (1985) who showed activation of human C_1 by reacting monoclonal antibody to C_1q

with its antigen. Similarly, reductions in acquired C_1-inhibitor could be shown to correlate with the presence of anti-idiotype antibody to monoclonal antibody (Geha *et al.*, 1985).

These are only a few of the possible immunochemical applications of monoclonal antibodies; however, standardization is one of the important factors in the adaptation of primary binding assays to diagnostic procedures in veterinary medicine.

MONOCLONALS AS *IN VIVO* REAGENTS FOR ANIMALS

The current or potential use of monoclonals in domestic animals can be categorized as follows (Shoemaker, Wall and Zurawski, 1977; Bigland, 1984; Spira *et al.*, 1985):

1. Passive antibody administered prophylactically or therapeutically for infectious diseases;
2. Passive antibody to target on particular cell markers, either alone or coupled to cytotoxic agents;
3. Passive antibody to enhance the clearance of toxic compounds;
4. Passive antibody to modulate the cellular or messenger components of the *in vivo* immune responses;
5. Anti-idiotypic antibody administered as an immunogen.

Although few of the above possible applications have gone beyond experimental use in small laboratory animals, there are some notable exceptions. The oral application of a monoclonal antibody directed to the K99 pilus antigen of enterotoxigenic *Escherichia coli* (ETEC) prevented severe fatal enteric disease in colostrum-fed and colostrum-deprived calves experimentally challenged with ETEC (Sherman *et al.*, 1983). This application has resulted in the marketing of a product but information regarding its commercial acceptance is not available. However, the variety of agents responsible alone or together in the pathogenesis of neonatal enteric disease probably means that monoclonal antibody 'cocktails' will be required where the causative agent(s) cannot be rapidly and economically defined. Investigations are in progress (S.D. Acres, personal communication) to determine the therapeutic value of orally administered monoclonal antibody to selected viral agents associated with neonatal bovine enteric disease.

The intravenous administration in sheep of a monoclonal antibody to bluetongue virus that neutralized the challenge strain prevented viraemia, the development of precipitating antibodies and clinical disease (Letchworth and Appleton, 1983a,b). The authors speculate 'that vaccines containing a single antigenic determinant might generate a similar neutralizing antibody and may both prevent bluetongue disease and perhaps interrupt virus transmission'. While a further report on this subject indicated that 'the monoclonal antibodies themselves are unlikely to be used commercially' (Letchworth, 1983), this would be an obvious application for anti-idiotypic antibody.

However, it remains to be shown whether the mouse monoclonal antibody will suffice or be preferable in this regard, or if interspecies or species-specific monoclonal antibodies are required, and whether or not these products will be more commercially viable than cloning the viral antigen.

Although there are many other reports on other applications of monoclonal antibody that are relevant to domestic animal diseases such as brucellosis (Montaraz *et al.*, 1986) they have not been evaluated in their natural host and are therefore best left to other reviews. Economics will undoubtedly be a major factor in determining the application of monoclonals to domestic animals of agricultural importance, while possibly having a lesser role in the companion animal sector. One would, none the less, expect rapid development in this regard, particularly if monoclonal technology can be extended to the development of species-specific reagents. Especially important will be the clarification of the biological function of particular isotypes and specificities in elucidating the pathogenesis of infectious diseases, mechanisms of resistance and the integration of the preceding into improved strategies of disease control (Pavlov *et al.*, 1982; Steplowski *et al.*, 1983; Greene *et al.*, 1985).

Anti-idiotype (Ab 2) has been used as an antigen to prepare anti-anti-idiotype (Ab 3) which has the same specificity as Ab 1 (*see Figure 7*). In other words, Ab 2 may be used as a vaccine to induce an antibody response to the original antigen. Thus Ab 2 was used to induce a response to transplantation antigens in mice (Bluestone *et al.*, 1981; Epstein *et al.*, 1982) and to produce protective immunity in mice against trypanosomes (Sachs, Esser and Sher, 1982; Sachs *et al.*, 1985). An anti-idiotype found in man to a hepatitis B epitope cross-reacted with anti-hepatitis B antibody from mice and six other species (Kennedy *et al.*, 1983a). Injection of this Ab 2 into mice increased the number of spleen cells secreting IgM anti-hepatitis antibody after challenge with hepatitis B antigen (Kennedy *et al.*, 1983b). Immunization with Ab 2 in the absence of hepatitis B antigen induced Ab 3 formation (Kennedy *et al.*, 1983b) and the Ab 3 was detectable in the serum (Kennedy, Melnick and Dreesman, 1984).

Similarly, Ab 2 has been made to neutralizing monoclonal antibody specific for rabies glycoprotein G and some of the Ab 2s, when used to immunize mice, resulted in a specific virus neutralizing antibody response (Reagan *et al.*, 1983). These data clearly indicate that Ab 2 can mimic an antigen epitope and cause the production of an immune response to it.

Monoclonal Ab 2 against monoclonal antibody specific for reovirus type 3 haemagglutinin (the viral-cell attachment protein) was shown to bind to the cell surface receptor and to inhibit the binding of reovirus type 3 to the cell (Kauffman *et al.*, 1983; Co *et al.*, 1985). This is another example of how antigenic mimicry by antibody may prove to be a useful tool in the future, not only to provide a mechanism of immunization in the absence of antigen but also as a probe for further study of cell receptors for viruses and their regulation. Immunoglobulin idiotypes for viral antigen–antibody systems have recently been reviewed by Kennedy and Dreesman (1985) and those for immune network have been reviewed by Köhler, Muller and Bona (1985).

Conclusion

Monoclonal antibodies are becoming established as important tools in veterinary medicine. This review has dealt with a few applications which demonstrate the versatility of monoclonal antibody, mostly but not exclusively in the diagnosis of disease. At this time monoclonal antibody has been applied to the detection of a wide variety of infectious agents of all classifications of micro-organisms, as well as to their indirect diagnosis by detection of their antibodies in body fluids by primary binding assays such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA).

Some of the problems and some of the advantages of monoclonal compared with polyclonal antibody have been addressed, as have some of the pitfalls in the use of this type of reagent. Other applications, including affinity purification of antigen, elucidation of the structure of antigens, the study of the cells of the immune systems, sex and pregnancy determinations and various immunochemical uses, especially cross-species fusion products, have been discussed.

Many reports of the uses of monoclonal antibodies in veterinary medicine have appeared in the literature over the past few years; however, application of these reagents is still in its infancy because of the large number of species under study, the number of tasks on hand and the lack of organized initiatives to standardize the reagents. These are, however, all difficulties that can be overcome in time with proper support.

The future potential for uses of monoclonal antibodies in veterinary medicine is outstanding. For instance, with monoclonal antibody as the detection system and the DNA probe for recognition there is no reason why embryos cannot be tested for the presence of specific pathogens in very small numbers, before they are transferred. Similarly, it is not impossible that cows could be used for production of ascites fluid—a veterinary immunologist's dream.

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Appendix A (reagents)

DULBECCO's MODIFICATION OF EAGLE's MEDIUM (DMEM)

With 4·50 gm/ℓ glucose and with 2 mM L-glutamine added weekly

Sp2/0 GROWTH MEDIUM

DMEM supplemented with 10% fetal bovine or horse serum (heat-inactivated at 56°C for 30 minutes);
1·25 mg gentamicin/100 ml

6-THIOGUANINE (100 × STOCK)

18·2 mg 6-thioguanine in 50 ml distilled water. Heat to dissolve. Filter sterilize and store frozen at −20°C.

PEG 50% (MW 1450)

The 50% (v/v) PEG solution is prepared on the day of fusion by adding 5 ml of DMEM to 5 ml PEG 1450 at 41°C. Adjust pH to 7·2 with 1·0 N NaOH and filter sterilize.

OXALOACETATE, PYRUVATE, INSULIN (OPI) (50 × STOCK)

750 mg oxaloacetic acid
22·7 ml sodium pyruvate (11 mg/ml)
40 mg bovine insulin.
Dilute with distilled water to 100 ml. Filter sterilize and store in plastic at −20°C.

ANTIBIOTIC-ANTIMYCOTIC MIX (100 × STOCK)

10 000 units/ml penicillin
10 000 μg/ml streptomycin
25 μg/ml fungizone.

HYBRIDOMA MEDIUM (HY)

DMEM supplemented with:
10% NCTC (inorganic salts, amino acids, vitamins, coenzymes and other components)
20% Fetal Bovine or Horse Serum (heat inactivated 56°C, 30 minutes)
2% OPI
1% antibiotic–antimycotic mix
1·25 mg gentamicin/100 ml

HYPOXANTHINE AND THYMIDINE MIXTURE (HT) (100 × STOCK)

To 60 ml distilled water add 136 mg hypoxanthine. Add 1·0 N NaOH until hypoxanthine is dissolved. Add 76 mg thymidine and readjust pH to 9·5 with acetic acid. Add distilled water to 100 ml. Filter sterilize. Store frozen at −20°C.

AMINOPTERIN (100 × STOCK)

To 60 ml distilled water add 1·8 mg aminopterin. Add NaOH to dissolve aminopterin. Adjust pH to 9·8. Dilute to 100 ml with distilled water. Filter sterilize. Store at −20°C.