MONOCLONAL ANTIBODIES IN ANIMAL PRODUCTION

their use in diagnostics and passive immunization

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Promotoren: dr. C.C. Oosterlee,
emeritus hoogleraar in de Veeteeltwetenschap,
Landbouwuniversiteit, Wageningen
dr. E.J. Ruitenberg,
hoogleraar in de Veterinaire Immunologie,
Faculteit der Diergeneeskunde, Rijksuniversiteit Utrecht

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P. Booman

MONOCLONAL ANTIBODIES IN ANIMAL PRODUCTION

their use in diagnostics and passive immunization

Proefschrift
ter verkrijging van de graad van
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op gezag van de rector magnificus,
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in het openbaar te verdedigen
op vrijdag 22 december 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

LANDBOUWUNIVERSITEIT
WAGENINGEN

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STELLINGEN

1. Het verkiezen van de produktie van monoclonale antistoffen boven die van polyclonale antistoffen berust vaak meer op gevoelsmatige en markttechnische argumenten dan op wetenschappelijke gronden.

   dit proefschrift

2. Het succes van de introductie van de melk-progesterontest op de markt is niet zozeer afhankelijk van de kwaliteit van de gebruikte monoclonale antistof alswel van de uitvoeringsvorm van de test; een één-stapstest zou recht doen aan de mogelijkheden die een dergelijke bepaling biedt.

   dit proefschrift

3. Absorptie-experimenten, waarin antistoffen tegen het H-Y antigen worden uitgeabsorbeerd met suspensies van ♂ en ♀ cellen, tonen aan dat er slechts een gradueel verschil in H-Y antigeen expressie is tussen de sexen.

   dit proefschrift

4. De resorptie van progesteron uit het vetweefsel bij het varken dient nader bestudeerd te worden als alternatief voor het algemeen aanvaarde mechanisme van compensatoire produktie door de ovaria na het wegvangen van progesteron uit de bloedbaan door antistoffen.

   Wang et al., 1984. J.Endocrin. 101:95-100; Cheesman and Chatterton, 1982. Endocrinology 111:564-571; dit proefschrift

5. Het niet aantoonbaar voorkomen van een anti-idiotype respons bij herhaalde toediening van homologe monoclonale antistoffen bij het rund ondersteunt de hapteen-carrier hypothese van Ehrlich et al. (1987).

   Ehrlich et al., 1987. Hybridoma 6:151-160; dit proefschrift

6. Afhankelijk van het aantal toedieningen, de dosis en de gewenste werkingsduur dient per toepassing op het gebied van immunomodulatie of immunotherapie een keuze gemaakt te worden tussen homologe of heterologe monoclonale antistoffen.

   dit proefschrift

7. Analoog aan de situatie bij de mens, dient toelating van monoclonale antistoffen voor passieve immunisatie bij landbouwhuisdieren nader geregeld te worden op grond van de bepalingen van de diergeneesmiddelenwet; eisen met betrekking tot verontreinigingen en toegevoegde stoffen vormen daarbij een noodzakelijk onderdeel.

8. Het onderzoek naar de geslachtsbeïnvloeding van pluimvee in een vroeg stadium van de embryonale ontwikkeling door middel van monoclonale antistoffen tegen het H-Y antigeen, bevindt zich in een ethisch spanningsveld vanwege enerzijds het ingrijpen in het diepste wezen van de natuur en anderzijds het alleen al in Nederland "euthaniseren" van 40 miljoen één-dagskuikens per jaar.

9. Popularisering van onderzoekresultaten kan gemakkelijk leiden tot onjuiste beeldvorming en zowel onderzoeker als samenleving schade berokkenen.

10. De visie dat de ontwikkelingen in de biotechnologie deel uitmaken van een wetenschap-technologie spiraal waaraan niemand richting geeft, doet tekort aan de wetenschappelijke en maatschappelijke verantwoordelijkheid van de onderzoeker.

11. In het wetenschappelijk onderzoek dient voorkomen te worden dat de wetenschappelijk-technische beheersing van geabstraheerde functies van het dier zodanig gaat overheersen, dat het dier zelf in zijn intrinsieke waarde en zin niet meer wordt erkend en enkel als produktiemiddel wordt gezien.
    n.a.v. Schuurman, 1989. Het 'technische paradijs', Kok, Kampen

12. Onverlet het streven naar het bevorderen van het welzijn der dieren, getuigt onze wetgeving van meer zorg voor dierenleven dan voor ongeboren mensenleven.

13. De snelle vorderingen op het gebied van de anthropogenetica kunnen gemakkelijk leiden tot een selectief aborteren van steeds meer ongeborenen met een steeds minder ernstige handicap.

14. In de discussies binnen de thema's van het Conciliair Proces: gerechtigheid, vrede en heelheid van de schepping zal scherper moeten worden onderscheiden tussen bijbels perspectief, politieke doelstelling en theoretische analyse, wil het Conciliair Proces tot praktische resultaten kunnen leiden.

15. Wetenschap geeft geen antwoord op de oorsprong en doel van het leven, wel op het proces.

Proefschrift van P. Booman
Monoclonal antibodies in animal production; their use in diagnostics and passive immunization

Wageningen, 22 december 1989

aan Anneke
Iris en Jurrien

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VOORWOORD

Dit proefschrift is voor een belangrijk deel tot stand gekomen door samenwerking met andere onderzoekinstellingen en bedrijven, waaronder het CDI in Lelystad, de Landbouwuniversiteit in Wageningen, de Veterinaire Faculteit in Utrecht, de University of California in Davis, Intervet International B.V. in Boxmeer en ImmuCell Corp. in Portland, Maine. Deze samenwerking, alsmede de brede opzet van het onderzoek, brengt met zich mee dat veel personen binnen en buiten het IVO bij het onderzoek betrokken zijn geweest. Graag wil ik dan ook een ieder bedanken die op enigerlei wijze heeft bijgedragen aan de realisatie van dit proefschrift. Ik hoop overigens dat mijn waardering voor al het gedane werk reeds in de dagelijkse omgang tot uitdrukking is gekomen. Toch wil ik nog enkelen hier met name noemen.

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Peter

CONTENTS

GENERAL INTRODUCTION 1

CHAPTER 1 Application of monoclonal antibodies in animal production: a review 3
(Livestock Production Science 18 (1988): 199-215)

CHAPTER 2 Production and characterization of anti-progesterone monoclonal antibodies for pregnancy diagnosis in cattle 25
(Innovations in Biotechnology (1984) pp. 259-266. E.H. Houwink and R.R. van der Meer, Editors. Elsevier, Amsterdam)

CHAPTER 3 Production and characterization of monoclonal antibodies against the H-Y antigen 37
(Journal of Reproductive Immunology 15 (1989): 195-205)

CHAPTER 4 Sexing bovine embryos with monoclonal antibodies against the H-Y antigen 53
(Livestock Production Science: accepted)

CHAPTER 5 Effects of anti-progesterone murine monoclonal antibodies on plasma progesterone concentrations and on anti-mouse reactions in cyclic pigs 73
(Journal of Reproduction and Fertility: submitted)

CHAPTER 6 Construction of a bovine-murine heteromyeloma cell line; production of bovine monoclonal antibodies against rotavirus and pregnant mare serum gonadotrophin 95
(Veterinary Immunology and Immunopathology: accepted)

CHAPTER 7 A comparative study on the use of bovine and murine monoclonal antibodies for passive immunization in cattle 117
(Veterinary Immunology and Immunopathology: submitted)

SUMMARY AND FINAL REMARKS 139

POPULAIRE SAMENVATTING 145

GENERAL INTRODUCTION

It has been some fourteen years since the experiments of Köhler and Milstein led to the first description of the development of monoclonal antibodies. Single molecular species antibodies with desired specificities could be produced by the fusion of antibody-producing cells with neoplastic cells. The original aims of Köhler and Milstein were to study the range of the antibody repertoire and to assess the contribution of somatic mutation to antibody diversity. The potential applications of these single monoclonal antibodies were not completely obvious then, but it was clear that they would be of significant value.

In fact, we now look back on the production of monoclonal antibodies as a discovery which has led to a vast range of applications, including analytical, diagnostic and therapeutic ones, covering the chemical, biological and medical sciences.

The present study aims to describe the potential usefulness of monoclonal antibodies in animal production. In Chapter 1 possible applications in the areas of diagnostics, passive immunization and fundamental research, with emphasis on reproduction, and the inherent limitations of the current hybridoma technology as well as the improvements that can be foreseen within the next few years, are discussed. Two examples of the use of monoclonal antibodies in diagnostics are extensively studied and highlighted. The first one is the production and characterization of monoclonal antibodies against progesterone for pregnancy diagnosis in cattle (Chapter 2). The second one is the production of antibodies against a male-specific protein, the H-Y antigen (Chapter 3). The anti-H-Y antibodies were applied in a simple, non-deleterious test for sexing bovine preimplantation embryos (Chapter 4). As an example of passive immunization, in Chapter 5 the possible immunomodulation of steroid plasma levels by anti-progesterone monoclonal antibodies in pigs has been described. The observed immunogenicity of murine monoclonal antibodies led us to develop a system for the production of monoclonal antibodies from domestic species, especially cattle (Chapter 6). More insight in the real necessity and the possible advantages of the use of homologous antibodies for passive immunization is desirable. In that context the availability of both murine and bovine monoclonal antibodies against a common antigen offered us the opportunity to compare the

efficacy of homologous versus heterologous antibodies when repeatedly administered to cattle (Chapter 7). The study is completed with a Summary and Final Remarks on some general aspects of the use of monoclonal antibodies in animal production.

CHAPTER 1

APPLICATION OF MONOCLONAL ANTIBODIES IN ANIMAL PRODUCTION: A REVIEW

P. Booman

Research Institute for Animal Production "Schoonoord",
P.O. Box 501, 3700 AM Zeist (The Netherlands)

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Abstract

The hybridoma technology for production of monoclonal antibodies circumvents many of the constraints associated with the use of conventional antisera, and consequently broadens the areas of application of antibodies in animal sciences. In the present review, the potential usefulness of monoclonal antibodies in animal production - with emphasis on reproduction - is discussed, including the inherent limitations of the current technology and the improvements that can be foreseen within the next few years. Because of their unique specificity and the fact that they can be produced in virtually unlimited quantities, monoclonal antibodies are an important tool in diagnostics. However, the use of these antibodies does not always guarantee absolute specificity, and the low affinity of many monoclonal antibodies will impose a number of limitations on their use. Monoclonal antibodies can also be used to optimize physiological processes such as growth and reproduction. For this, homologous antibodies will probably offer several advantages over their murine counterparts in terms of effectiveness for passive immunization. Some success has already been achieved in the development of monoclonal antibodies from livestock species. Finally, it is shown that monoclonal antibodies are becoming extremely powerful research tools.

Introduction

When an antigen is introduced into an animal, one aspect of the immune response is the secretion by plasma cells of antibodies: immunoglobulin molecules with binding sites that recognize the shape of particular determinants on the surface of the antigen and bind to them. The combination of antibody with antigen sets in train processes that can neutralize and eliminate the antigen.

Quite apart from the natural function of antibodies in the immune response, they have long been an important tool for investigators, who capitalize on their specificity to identify or label particular molecules or cells and to separate them from a mixture. However, the heterogeneity of the immune response has complicated the use of antibodies as reagents. If the antigen is a complex macromolecule, such as a protein, it will con-

tain many antigenic determinants. Each of these antigenic determinants may trigger specific B-lymphocytes to differentiate and generate clones of plasma cells secreting antibody. Furthermore, a single antigenic determinant can stimulate multiple B-cell clones synthesizing antibodies that vary in specificity and affinity.

Conventional antiserum production

Immunization with most antigens results in a polyclonal response and the accumulation of many different antibodies in the serum. Antisera, therefore, may contain immunoglobulins of varying isotypes, affinities, specificities and biological activities. The quantity and quality varies from animal to animal and even from one bleed to the next in a given animal. This situation is made more complex if the immunizing agent contains minor contaminants of a highly immunogenic nature that are capable of inducing large amounts of an irrelevant antibody. Finally, good antisera are usually available in limited amounts, especially if they have been raised against weak antigens or if it has been necessary to absorb the antiserum to make it specific. Consequently, the production and standardization of specific immunological reagents is a difficult task.

Hybridoma technology

The hybridoma technology developed by Köhler and Milstein (1975) makes it possible to overcome these difficulties and, in many cases, to obtain a serological reagent that is not only homogeneous and predictable, but is also tailormade to the needs of each of the many applications of immunological reagents (for a comprehensive review, see Milstein, 1980).

The basis of hybridoma technology is the immortalization of B-lymphocytes with antibody-producing capacities, but limited in vitro growth characteristics. The lymphocytes are fused with cells from a non-antibody-producing and continuously growing tumour cell line or myeloma cell line so that hybrids continue to secrete antibodies while gaining the immortality of the parent tumour cell. Because each B-lymphocyte produces an immunoglobulin molecule with a fixed specificity, clones derived from hybridized cell populations are homogeneous in nature, and each of the clones secretes an immunoglobulin with a single molecular structure and antigen specifici-

ty. Thus, all the antibody molecules secreted by the same cell line exhibit identical specificity, affinity and isotype (class or subclass). The antibodies produced by the fusion procedure are known as monoclonal antibodies, distinguishing them from the heterogeneous polyclonal antibodies obtained by conventional antibody production. Antibody-producing clones may be stored in liquid nitrogen in the viable state and retrieved at will for culture and production of the corresponding antibody. In Fig. 1, a comparison is made between the monoclonal antibody technique and the conventional technique to prepare antibodies.

There is a number of excellent books and reviews dealing with the specific details of monoclonal antibody production techniques (Goding, 1980, 1983; Galfrè and Milstein, 1981; Campbell, 1984). The benefits of monoclonal antibodies have resulted in the hybridoma technology being applied to many basic and practical problems. In animal production, monoclonal antibodies are increasingly finding application in the areas of diagnostics, passive immunization and fundamental research. In the following sections, some applications of monoclonal antibodies within these areas with emphasis on reproduction are discussed. A complete review is beyond the scope of this article. The aim is to describe critically the potential usefulness of monoclonal antibodies in animal production, the inherent limitations of the current hybridoma technology and the improvements that can be foreseen within the next few years.

Monoclonal antibodies versus polyclonal antibodies

It is evident that monoclonal antibodies have some major advantages over conventional polyclonal antisera. Firstly, monoclonal antibodies are homogeneous and permanently-available reagents that can be obtained in large amounts. Another major advantage is that pure antibodies can be obtained even when impure antigens were used to immunize. This is important because many biologically-significant antigens have not been purified to homogeneity. It is even possible to use the hybridoma technology to look for antigens whose existence is only suspected, and whose structure and properties are completely unknown. In addition to these obvious benefits,

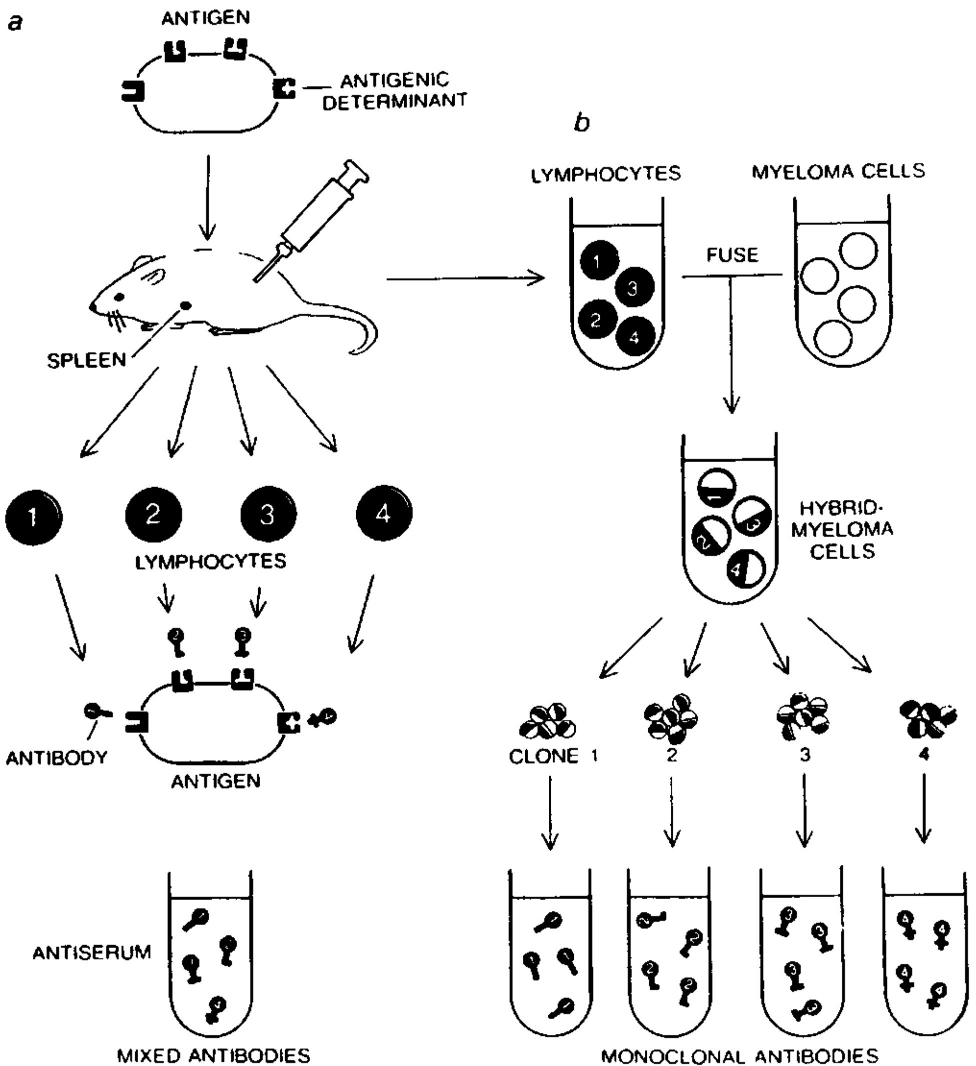

Fig. 1. Immune response is initiated (a) when an antigen molecule carrying several different antigenic determinants enters the body of an animal. The immune system responds: lines of B-lymphocytes proliferate, each secreting an immunoglobulin molecule that fits a single antigenic determinant (or a part of it). A conventional antiserum contains a mixture of these antibodies. Monoclonal antibodies are derived by fusing lymphocytes from the spleen with malignant myeloma cells (b). Individual hybrid cells are cloned, and each of the clones secretes a monoclonal antibody that specifically fits a single antigenic determinant on the antigen molecule (Milstein, 1980).

hybridoma technology makes it possible to generate a battery of monoclonal antibodies that react with a particular antigen and to select those antibodies that will be best for a particular task. Individual monoclonal antibodies can be chosen on the basis of their affinity, specificity or isotype.

Despite the great potential of monoclonal antibodies, there are several disadvantages to their use that deserve consideration. Since a monoclonal antibody detects a single antigenic determinant it may be more influenced by conditions that alter the binding properties of the single binding site, and in addition it will not form the lattice-like antigen-antibody structure required for direct precipitation, unless the antigen has more than one identical antigenic determinant (i.e., polyvalent antigen) distributed in a favourable conformation. The utility of otherwise valuable monoclonal antibodies may also be limited by the fact that the antibodies are of a specific subclass and, therefore, restricted to the functions that can be carried out by antibodies of that class. Such limitations may be minor and can often be circumvented, but must be taken into consideration when these reagents are used instead of polyclonal antisera.

Diagnostics

Monoclonal antibodies in diagnostics

Several recent reviews (Scott, 1985; Sevier, 1985) have expressed the belief that monoclonal antibodies will soon replace polyclonal antisera as standard reagents for immunological assays. However, the time and effort required to obtain monoclonal antibodies with suitable properties is considerable. Furthermore, for those assays which already operate satisfactorily with adequate supplies of conventional antiserum there may be little to be gained by production of a monoclonal antibody.

The properties of monoclonal antibodies mean that they are very valuable diagnostic tools in certain situations. Firstly, it is possible to produce specific antibodies from impure material. Secondly, for different antigens which possess common structural features, antibodies can be obtained which are directed against structures unique to a particular antigen and are, therefore, totally specific. Finally, the capacity to produce

monoclonal antibodies in large quantities and with constant characteristics is valuable for assays demanding substantial amounts of antibody and for standardizing immunological assays.

Pregnancy diagnosis

An example of a typical diagnostic test in animal production in which monoclonal antibodies might be used is the milk-progesterone test for confirmation of oestrus and pregnancy diagnosis in cattle (Robertson and Sarda, 1971; Heap et al., 1973). The progesterone concentration in either blood plasma or in milk at oestrus is very low and rapidly rises to a high level at mid-cycle. This level is maintained throughout pregnancy. If a cow does not become pregnant, the progesterone level falls around Day 16-19 after last oestrus. Several enzyme immunoassays for the determination of progesterone have been described in the literature, most of them employing polyclonal antibodies (Arnstadt and Cleere, 1981; Van de Wiel and Koops, 1986). However, for commercialization, the milk-progesterone test has to be standardized and in that case monoclonal antibodies are the preferred reagents. Several groups have prepared monoclonal antibodies to progesterone (Fantl et al., 1982; White et al., 1982; Booman et al., 1984a). These antibodies differed considerably in their specificity and affinity for progesterone. The best monoclonal did not detect progesterone with any greater sensitivity than the conventional polyclonal sera. However, the use of monoclonal antibodies leads to improvements in test standardization and avoids the dependency upon animals producing high quality antisera. ImmuCell Inc. (U.S.A.) has designed a rapid progesterone cow-side test based on the antibody with the best characteristics produced in our laboratory. This test can be performed by the farmer himself and the results are known within 4 min.

In pigs, increased levels of oestrone sulphate in plasma have been demonstrated early in pregnancy from Day 16 with a maximum on Day 40 (Robertson and King, 1974). A pregnancy diagnosis test can be based on the differences in plasma levels between pregnant and cyclic pigs (Guthrie and Deaver, 1979; Edqvist et al., 1980). Besides standardization of a kit for pregnancy diagnosis, monoclonal antibodies have the advantage that they can specifically bind with oestrone sulphate whereas polyclonal antibodies are mostly directed against oestrone. The necessity for hydrolysis

of oestrone sulphate can, therefore, be avoided. At our laboratory very specific and high affinity monoclonal antibodies have been prepared for oestrone and oestrone sulphate, respectively.

A similar application of monoclonal antibodies is their use in rapid solid phase sandwich enzyme immunoassays to measure equine and bovine luteinizing hormone for detection of ovulation and equine pregnant mare serum gonadotrophin (PMSG) for pregnancy diagnosis (Kasper et al., 1985a,b; Roser et al., 1985). These monoclonal antibodies could be useful in manufacturing test kits for field applications demanding substantial amounts of antibody.

Sexing embryos and sperm

Another application in animal reproduction is the use of monoclonal antibodies against the H-Y antigen for sexing bovine embryos before transplantation (for a review, see Booman, 1986). The H-Y antigen is a histocompatibility antigen, detected in 1955 by Eichwald and Silmser, and present on the surface of male cells, but not on those of the female (Billingham and Silvers, 1960). Because of the exclusive presence of the H-Y antigen in the mammalian male (Wachtel et al., 1975) and its detection early in embryonic development (Krco and Goldberg, 1976), it has become possible to predict the phenotypic sex of the offspring on the basis of embryonic H-Y antigen expression. Polyclonal antibodies against the H-Y antigen can be raised by injecting female C57Bl/6 mice with cells of males of the same inbred strain. Anti-H-Y antibodies of the mouse have been used to identify XY cells in some 70 species from all classes of vertebrates (for references, see Wachtel, 1983). The antigen appears to lack species specificity and, therefore, the antibodies can be used to identify bovine embryos. The H-Y antigen is a weak antigen and immunization with H-Y antigen usually results in production of low titer, low affinity antisera. Because only a low percentage of mice have a good antibody response and their sera run out quickly, it would be preferable to have monoclonal antibodies. Several monoclonal antibodies have already been developed (Koo et al., 1981; Farber et al., 1982; Shapiro and Goldberg, 1984). However, the affinity of all these monoclonal antibodies appears to be low. In our laboratory we have been trying to produce specific, high affinity monoclonal antibodies against the H-Y antigen (Booman et al., 1988a). Evaluation of bovine embryos for expression of H-Y antigen using these monoclonal antibodies in

an indirect immunofluorescence assay has been shown to be reasonably accurate. The assessment of fluorescence, however, is highly subjective. Work must still be done to modify the procedure in such a way that subjectivity is reduced.

It is unlikely that monoclonal antibodies against the H-Y antigen can be used as a means for the selection of X or Y chromosome-bearing sperm. Success would depend on haploid expression of H-Y antigen by Y-bearing spermatozoa. It is clear, however, that expression of the H-Y antigen requires the Y-linked gene as well as an X-linked one (Ohno, 1979; Wachtel and Ohno, 1980). Ohno (1982) supposed that the H-Y antigen present in abundance on the sperm plasma membrane is actually contributed by Sertoli cells and not synthesized by germ cells with a dormant X chromosome, the implication of which is that spermatozoa passively become H-Y positive regardless of sex chromosomal type.

Other diagnostic applications

A valuable in vitro diagnostic assay might be to evaluate whether sperm has been capacitated or not. Capacitation is the alteration of factors on the sperm cells necessary for fertilization (for a review, see Bedford, 1983). Monoclonal antibodies specific for appropriate surface antigens may give information about changes in surface organization that appear to accompany capacitation (Saxena et al., 1986). Preservation of boar semen during several days in appropriate buffer may not induce capacitation; on the other hand, for in vitro fertilization purposes capacitated sperm is a prerequisite. Such antibodies require a high degree of specificity.

Specificity is also of importance in the production of blood-typing reagents for farm animals. Blood group antigens have been widely used in the genetic identification of livestock, and the antigens of the major histocompatibility complex hold promise as markers for immune response genes in domestic species. Monoclonal antibodies would increase the efficiency of routine blood typing and allow for international standardization of genetic markers of many types (Tucker et al., 1981).

Another potentially valuable application is in the area of food analysis, such as the identification of the species origin of meat products and the determination of residues of hormones or growth promotors in milk and meat (for references, see Morris and Clifford, 1985). Monoclonal anti-

bodies would improve the accuracy and reproducibility of the results from immunoassay techniques and offer a cheap standardized method of screening a large number of samples rapidly and accurately.

Specificity and affinity of monoclonal antibodies

Two remarks should be made concerning the use of monoclonal antibodies in diagnostics. In polyclonal antisera where antibodies are derived from multiple distinct B-cell clones, cross-reactivity is a major problem. Although the technique of monoclonal antibody production selects for an antibody with specificity for a single antigenic determinant, this does not always preclude the presence of cross-reactivity. The specificity of an antibody is a quantitative phenomenon that is determined by its affinity for a defined antigenic structure as compared to other antigens. It is clear that the discriminating abilities of monoclonal antibodies are potentially very great, although impossible to predict in advance. The ability to obtain highly-specific antibodies obviously depends on the existence of structural features unique to a given molecule, and on the maximum affinity differences likely to be possible to allow binding to these, but not to modified determinants on other molecules. It is also necessary that such structural features make a significant contribution to the overall immunogenicity of the molecule, although even in this case it is a matter of generating enough monoclonal antibodies to find the right one.

In addition, most antibodies derived by hybridoma technology have affinities far below the corresponding conventional antisera. In a polyclonal antiserum, the majority of antibodies will also have low affinities, but the few high affinity antibodies will dominate the reaction and provide the necessary sensitivity. However, a monoclonal antibody can exhibit an affinity anywhere within the full range and one must identify those few of sufficient affinity. Some recent evidence has suggested that the high affinity of polyclonal relative to monoclonal antibodies may reflect more than a simple statistical incidence of different individual affinities in a large population. Some mixtures of 2 monoclonal antibodies for different antigenic determinants on a given antigen show cooperativity in that the affinity of the mixture is considerably higher than that of the individual antibodies (Ehrlich et al., 1983). If high affinity monoclonal antibodies are of particular importance, the screening assays for selecting

relevant antibodies must be designed to exclude low affinity antibodies. All things considered, it is always necessary to evaluate carefully the need of making monoclonal instead of polyclonal antibodies for a given antigen as considerable time and effort is required to obtain monoclonal antibodies with suitable properties.

Passive immunization

Immunoneutralization by active and passive immunization

Immunization against biologically active substances, such as hormones, can produce a wide variety of effects, including neutralization of the selected substance, blocking of action or even enhancement of action of a substance. The nature of these effects will be influenced by level, specificity and affinity of the antibodies, regardless of whether they are a result of active or of passive immunization.

It has been hypothesized (Cox et al., 1985) that in the neutralization of selected substances (the system that is most commonly considered), the active substance is bound to antibody and hence becomes biologically unavailable. In this way, there is a direct reduction of biological material available for target tissues. Many other, secondary, effects may be a consequence of the immunization, e.g., disturbance of a metabolic network and quantitative alteration in other active substances. There are now many examples showing the physiological effects of immunizing animals against hormones; for instance, immunization of sheep to gonadal steroids during the breeding season may raise the ovulation rate (Pathiraja, 1982) resulting in an increased number of lambs born (Land et al., 1982). A possible mechanism that can explain such an effect is that the antisera interfere with the equilibria between gonadotrophin release and gonadal activity (Land et al., 1983). Immunization against luteinizing hormone-releasing hormone can produce immunocastration and meat quality changes in rams and bulls (Schanbacher, 1982; Robertson et al., 1982); somatostatin immunity has been reported to bring about growth changes (Spencer et al., 1983) and immunization against inhibin results in early onset of puberty as well as increased ovulation rate in sheep (O'Shea et al., 1982; Henderson et al., 1984).

Alternatively, there is a possibility that antibodies could be used effectively to bind to receptors and hence to block hormones from reaching the receptors, thus resulting in modification of hormone action.

Enhancement of action of a substance by immunization has hardly been explored, but may have very useful applications. Such effects were noted by Holder et al. (1985), who showed that the binding of a monoclonal antibody to human growth hormone resulted in marked enhancement of the somatogenic activity of the hormone in vivo. The same group found that the binding of some monoclonal antibodies to bovine growth hormone also resulted in enhancement of biological activity and that the effect was dependent on the binding site specificity of the monoclonal antibody (Aston et al., 1987). The mechanisms behind this phenomenon are not clear.

Immunoneutralization by active immunization is not a reproducible effect. Within species, and even within breeds, there can be marked variations in the magnitude and speed of antibody response. The effect of active immunization is manifested only in those animals which form adequate titers of antibodies. The effects could be brought about in a repeatable manner by passive administration of preformed antibodies. Also, in contrast to the slow acquisition of antibody titer following active immunization, passive immunization results in an immediate neutralization. The ability to immediately and specifically neutralize hormone activity provides a means of influencing a physiological process at a very precise moment. A good example of this is the administration of (polyclonal) antibodies against pregnant mare serum gonadotrophin (PMSG) in superovulated cattle immediately after insemination, where the surplus of PMSG is neutralized and consequently the number of embryos of good quality can increase (Bouters et al., 1983). Finally, passive immunization has proved useful in the treatment of certain animal diseases where prophylactic measures are not possible. However, the amounts of antisera required for passive immunization are very large, so that with polyclonal antibodies it is possible to treat only a restricted number of animals. The hybridoma technique offers the possibility of obtaining antibodies of the required specificity and characteristics in unlimited amounts.

The first commercial application of monoclonal antibody administration in vivo was to protect neonatal calves against diarrhoea caused by enterotoxigenic *Escherichia coli*. Oral administration of monoclonal antibodies specific for the K99 pilus antigen of *E. coli* passively protects animals

in laboratory and field conditions (Sherman et al., 1983). Recently, a commercial preparation of monoclonal antibodies has been introduced against PMSG to improve the results of superovulation in cattle (Dieleman et al., 1988). Other applications will certainly follow.

Limiting factors of monoclonal antibodies in passive immunization

It will, however, take some time for the potential of monoclonal antibodies to be fully realized in animal production. In spite of new mass culture techniques, the production costs of purified monoclonal antibodies are still high so that not all applications are yet economically attractive. Another limitation may be the reaction of the treated animal to the effects of passive immunization. For instance, in the case of anti-steroid antibodies, the animal may restore its hormone level via compensatory production, shifting of equilibria in the steroid pathway and release of steroids from fat deposits (Booman et al., 1984b; Wang et al., 1984). A third limitation might be the murine or rat origin of monoclonal antibodies when applied in other species. Once these antibodies have been recognized as foreign, an anti-mouse immunoglobulin response may develop which limits the further effectiveness of the monoclonal antibody. In addition, heterologous antibodies may not elicit cooperative cellular effects. As reported by Nose and Wigzell (1983) and reviewed by Larrick and Buck (1984), antibodies have species-specific carbohydrates which are important in several antibody effector functions.

Despite these drawbacks, murine monoclonal antibodies have been used succesfully to neutralize PMSG in superovulated cattle (Dieleman et al., 1988). It is not yet known, however, whether repeated injections given over a period of time remain effective. In pigs, repeated administration of murine antibodies generated a significant antibody response to mouse immunoglobulins (Arriëns and Booman, 1988). Human clinical trials with murine monoclonal antibodies indicate that almost all non-immunocompromised patients developed anti-mouse immunoglobulin antibodies which neutralized the therapeutic effect (for references, see Cole et al., 1985). The neutralizing effect of human anti-mouse antibody is due to a host immune response against both the constant portions of the mouse immunoglobulin molecule and the variable region (idiotype) reacting with the antigenic determinant (Levy et al., 1984).

These findings have important implications for the eventual use of homologous monoclonal antibodies for passive immunization. Such antibodies may likewise evoke an anti-idiotypic immune response. However, Ehrlich et al. (1987) reported that only one out of five rhesus monkeys injected repeatedly with human monoclonal antibodies produced anti-idiotypic antibodies. They suggested that the anti-idiotypic antibodies that are prevalent in the human anti-mouse monoclonal antibody response are elicited through a hapten-like effect, in which the heterologous immunoglobulin acts as a carrier for the idiotype.

Production of homologous antibodies

Although anti-idiotypic responses cannot be excluded as a complicating factor, homologous antibodies will probably offer several advantages over their murine counterparts in terms of effectiveness for passive immunization. Unfortunately, the production of, for instance, bovine or porcine monoclonal antibodies has been greatly hampered by the lack of myeloma fusion partners for lymphocytes from these species.

Nevertheless, some success has already been achieved in the development of monoclonal antibodies from livestock species. Srikumaran et al. (1983) demonstrated the potential of interspecies hybridomas, produced by fusing mouse myeloma cells with bovine lymphocytes. This strategy circumvents the necessity of developing homologous fusion partners. More recently, the generation from such interspecies fusions of bovine antibodies with specificity for bovine enteric coronavirus (Raybould et al., 1985) as well as antigen-specific ovine monoclonal antibodies has been reported (Beh et al., 1986; Groves et al., 1987). A severe limitation to interspecies hybridomas, however, is their genetic instability, due to the selective elimination of the non-murine chromosomes.

In another approach, mouse x bovine hybrid myelomas (heteromyelomas) have been constructed in attempts to obtain a better fusion partner for the production of bovine monoclonal antibodies (Tucker et al., 1984; Booman et al., 1988b). It was anticipated that a heteromyeloma would retain the superior fusion characteristics of the mouse myeloma cells and be better able to support stable bovine antibody production because of the presence of bovine chromosomes. With these heteromyeloma cell lines bovine antibodies have been produced against the Forssman antigen (Tucker et al.,

1984) and at our laboratory against rotavirus and PMSG. Current research is focused on the effectiveness of murine monoclonal antibodies compared to bovine antibodies against PMSG in superovulated cattle after repeated treatments.

Fundamental research

The potential of monoclonal antibodies as research tools is clearly enormous. This is due to the specificity of monoclonal antibodies which allows discrimination between closely related antigenic determinants.

One of the areas where monoclonal antibody technology is having a major impact is in analysis of the mechanism of immune regulation and delineation of the genetic basis of disease susceptibility. The use of monoclonal antibodies has permitted the development of precise immunological reagents that can be used to define the network of functional subpopulations of lymphoid cells in livestock species and the antigens of the major histocompatibility complex that are centrally involved in the regulation and expression of the immune response (for references, see Davis et al., 1985). Monoclonal antibodies can be applied to define the functional roles of sperm constituents during differentiation and fertilization and to identify and resolve the functions of many regulatory substances with crucial roles in mammalian gametogenesis, fertilization and development (for references, see Bellvé and Moss, 1983). A further consequence of the fact that one particular monoclonal antibody recognizes only one antigenic determinant of an antigen is that monoclonal antibodies may be useful reagents in molecular studies of receptor structure and function (Greene et al., 1980) or in studying the orientation of hormones when bound to their receptors (Moyle et al., 1982).

Monoclonal antibodies can also be used effectively as biochemical reagents for the affinity purification of antigenic molecules of interest in animal production. The affinity of monoclonal antibodies is frequently reported as being low relative to their polyclonal counterparts. Low affinity antibodies are generally more suitable than high affinity antibodies for use in immunopurification schemes (Morgan et al., 1984), because of the relative ease of elution of antigen in an active form.

A novel and useful application of monoclonal antibodies is in the devel-

opment of anti-idiotypic antibodies. The binding site on the monoclonal antibody, the idiotype, consists of a region complementary to the antigenic determinant. The production of antibodies directed against another antibody molecule will result in some antibodies specific for the idiotypic region, the so-called anti-idiotypic antibodies (Marx, 1985). The binding region of the anti-idiotypic antibody mimics the architectural configuration of the original antigenic determinant. The mimicry is so accurate that in some cases the anti-idiotypic antibody may be utilized instead of antigen for the identification or induction of antibody. It has been suggested by Kelley and Lewin (1986) that this approach could be particularly useful in vaccine production for antigen preparations that might remain infectious after isolation, or for those antigens that are difficult to isolate and to characterize. Anti-idiotypic antibodies might also be used as a tool to mimic the antigen in vitro (e.g., diagnostic assays) or in vivo (e.g., to mimic insulin and cause glucose entry into a cell).

These examples are only intended to give an impression of the many possible uses of monoclonal antibodies in fundamental research and thus to show what a valuable instrument has been made available to animal sciences.

Conclusions

The particular advantages of monoclonal antibodies can be first and most easily shown in immunodiagnosis. Once a hybridoma producing a monoclonal antibody appropriate for a particular task has been obtained, large amounts of a homogeneous and reliable reagent are available for as long as they are needed. Monoclonal antibodies may also prove to be important in passive immunization, although such applications require more basic research. The advent of better methods for producing antibodies from livestock species may increase the versatility of this technology. Finally, it is evident that monoclonal antibodies are becoming extremely powerful research tools. There is no doubt that the monoclonal antibody technology will have an important impact on the improvement of animal quality and productivity.

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CHAPTER 2

PRODUCTION AND CHARACTERIZATION OF ANTI-PROGESTERONE MONOCLONAL ANTIBODIES FOR PREGNANCY DIAGNOSIS IN CATTLE

P. Booman, M. Tieman, D.F.M. van de Wiel, J.M. Schakenraad and W. Koops

Research Institute for Animal Production "Schoonoord",
P.O. Box 501, 3700 AM Zeist (The Netherlands)

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Abstract

In order to standardize an enzyme immunoassay of milk-progesterone as a routine method for early pregnancy diagnosis in cattle, several monoclonal antibodies against progesterone were produced. The antibodies differed considerably in their binding affinity for progesterone and showed distinct specificities for a variety of steroids. However, the antibody with the highest association constant for progesterone ($K_A = 2 \times 10^{10}$ l/mol) also showed good specificity. Using this antibody in a direct double antibody solid phase enzyme immunoassay (EIA), the detection limit of the assay was 1 pg, whereas a 50% reduction of maximum binding in the standard curve was obtained by addition of 18 pg of progesterone. The titer (= working dilution in the EIA-system) of ascites fluid of this IgM antibody was 1:13,500,000, and of cell culture medium 1:12,000.

Introduction

The day-to-day variations in plasma progesterone in non-pregnant cows display a cyclic pattern, which is characterized by minimal plasma progesterone levels from two days before to three days after ovulation, a sharp rise from Day 5 to Day 7 of the cycle, and a very slow rise or plateau on the subsequent eight to ten days.

An important feature of the bovine cycle is the abrupt fall in peripheral plasma progesterone levels which occurs between the third and second day before oestrus. Since this fall is not seen in pregnant animals, an early pregnancy diagnosis can be made at 3 weeks after insemination by determination of progesterone levels in plasma or milk. Progesterone levels in milk accurately reflect the concentration in plasma. It is preferable to measure progesterone in milk because milk samples can be easily collected. Application of the milk-progesterone test for the purpose of early pregnancy diagnosis can be of great importance to the modern dairy farmer with a large herd and a tight calving pattern, where the optimum calving interval has a pronounced financial impact.

For the direct measurement of progesterone in milk and plasma, a sensitive and simple enzyme immunoassay (EIA) has been developed by Van de Wiel and Koops (1). Routine application of the test in practice and on

a large scale demands substantial amounts of antibody. Unlimited quantities of identical antibodies, which will allow standardization of the assay within and between laboratories, can be produced by the technique of somatic cell fusion according to Köhler and Milstein (2). Improvement in assay sensitivity and specificity can be obtained by screening a number of cell lines for the production of monoclonal antibodies with desired characteristics. In this paper we describe the production of monoclonal antibodies to progesterone by the hybridoma technique, and the use of one of these antibodies for the measurement of progesterone by EIA.

Materials and Methods

Production of hybridomas

Hybridoma cell lines were produced according to techniques initially developed by Köhler and Milstein (2). 6-8 Week old male Balb/c mice were injected intraperitoneally with either 11α-hydroxyprogesterone-hemisuccinate conjugated to bovine serum albumin (P-11α-BSA), or progesterone-7α-carboxyethylthioether conjugated to bovine thyroid globulin (P-7α-BTG). 100 μg of each conjugate had been dissolved in phosphate buffered saline (PBS) and emulsified with complete Freund's adjuvant (1:1). A second injection was given three weeks later in exactly the same way as above, except that incomplete Freund's adjuvant was used. Finally, 6-10 weeks after the last inoculation and four days before collection of the spleen cells, the animals were boosted intravenously with 50 μg of antigen in 0.1 ml PBS.

Spleen cells were isolated for fusion and erythrocytes were lysed by incubation with 0.83% (w/v) NH_4Cl for 1 min at room temperature (rT). The X63-Ag8.653 mouse myeloma cell line (3) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum (FCS)(DMEM-15), penicillin, streptomycin and glutamin.

For each fusion a mixture of 10^8 spleen cells and 2.5×10^7 myeloma cells was washed three times in DMEM. The pellet was resuspended in 1 ml of 50% (w/v) polyethylene glycol 4000 in DMEM containing 10% dimethylsulfoxide. The suspension was incubated for 1 min at rT and subsequently diluted stepwise over a period of 6 min with 8 ml DMEM. Then 6 ml DMEM-15

were added and the suspension was pipetted up and down gently and centrifuged for 7 min at 800 rpm. The pellet was resuspended in 150 ml DMEM-15 and the cells were distributed over six 384-well Greiner® TC cloning plates and incubated at 37°C in a humidified CO_2-incubator (7.5% CO_2) for 24 h, with 5 x 10^4 mouse macrophages per ml as feeder cells. The cultures were then supplemented with an equal volume of DMEM-15 containing hypoxanthine, aminopterin and thymidine (HAT). Medium was refreshed every 3-4 days for 2 weeks with HAT medium with decreasing concentrations of FCS. Then the culture medium was exchanged for DMEM containing hypoxanthine and thymidine only. Selected antibody-producing clones were passaged onto tissue culture flasks and split for storage in liquid nitrogen. Cloning of the hybrid cells was done by limiting dilution in 96-well plates in a feeder cell culture-conditioned medium.

For production of ascites fluid, Balb/c mice were injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane). After 1-3 weeks the mice were inoculated with 2.5 x 10^6 hybridoma cells. About 0.5 ml of serum and 2-8 ml of ascites fluid were obtained from individual animals between 1 and 3 weeks later.

Detection of antibody production

Hybridoma supernatants and ascites fluids were screened for the presence of anti-progesterone antibody in a double antibody solid phase EIA. The Ig fraction of a rabbit antiserum against mouse immunoglobulins was isolated by Na_2SO_4 precipitation and was used for coating (18 h at 4°C) of polystyrene microtiter plates (10 μg Ig/ml, 100 μl/well) at pH 9.6 in 0.05M $NaHCO_3$ buffer. A second coating (50 min at rT) was done with 1% BSA to prevent non-specific binding effects. The plates were washed, dried, sealed and stored at 4°C until use. Subsequently the plates were incubated (2 h at rT) with 100 μl hybridoma supernatant or ascites fluid diluted in 0.04M PBS pH 7.2 containing 0.1% BSA. The supernatants were aspirated and the plates were washed five times with 0.05% Tween 80. Synthesis of the conjugates was performed according to the modified mixed anhydride method of Dawson et al. (4). Horse-radish peroxidase was conjugated to either 6β-hydroxyprogesterone-hemisuccinate, 7α-hydroxyprogesterone-carboxyethylthioether or 11α-hydroxyprogesterone-hemisuccinate (P-6-HRP, P-7-HRP and P-11-HRP, respectively). P-HRP was added (5 ng/well) and the

plates were incubated for 2 h at rT in the dark. The reaction was stopped by aspiration of the supernatants and the wells were washed five times with 0.05% Tween 80. Substrate solution was prepared in 0.1M acetate-citrate buffer pH 5.5 and contained tetramethylbenzidine in dimethylsulfoxide (0.0075%) and H_2O_2 (0.004%). 150 μl of substrate solution was added to the wells of the plates. After an incubation period of 45 min at rT, the reaction was stopped with 50 μl 4N H_2SO_4. The absorbance was read at 450 nm with a spectrophotometer (Titertek multiskan).

Characterization of antibodies

The specificity and the affinity of the various monoclonal antibodies in the supernatants of the cell cultures were determined according to Abraham (5) and Scatchard (6), respectively. The assays were performed as described for the screening system of antibody production by the hybrid clones. The titer of the antibodies was defined as the dilution of cell culture supernatant or ascites fluid which resulted in half of the maximum binding of 5 ng of tracer.

Immunoglobulin (sub)class was determined by the double diffusion method of Ouchterlony. Anti-mouse immunoglobulins were obtained from Miles Laboratories.

Results and Discussion

Production of hybridomas

Fusion of spleen cells from immunized mice with the mouse myeloma cells resulted in the growth of hybrid cells in about 60% of the wells of the TC cloning plates. After two weeks of growth specific antibody-secreting cells were detected by EIA in about 10% of the wells with hybrid clones. Although the cells were cloned as soon as possible, more than half of the positive hybridomas lost their ability to secrete antibodies during subsequent cell culture. Two cell fusions with P-11α-BSA as immunogen and three cell fusions with P-7α-BTG as immunogen ultimately resulted in 6 and 8 stable cell lines, respectively. No significant difference in the frequency of hybridomas was observed between the fusing experiments with the two

different antigens.

Characterization of monoclonal antibodies

Specificity and affinity of the monoclonal antibodies, as determined according to Abraham (5) and Scatchard (6) respectively, are shown in Table 1A and 1B.

The cross-reactivity of the antibodies produced by the different cell lines varied widely. The high degree of cross-reactivity with 11α-hydroxyprogesterone and 11β-hydroxyprogesterone for antibodies raised against the 11α-hydroxyprogesterone conjugate was not unexpected. The highest cross-reactivity with 11β-hydroxyprogesterone (24,194%) was observed in cell line 1ab4, which had a very low affinity for progesterone (K_A = 2 x 10^7 l/mol) and a poor specificity. Most antibodies showed remarkably high cross-reactivity with pregnanedione, 11-ketoprogesterone, 5-pregnene-3β,20α-diol and, except for the cell lines 1ab4, 2VIII and 7II, with 5α-pregnane-3,20-dione. Some antibodies bound to these steroids with comparable or even higher affinity than to the homologous steroid hapten. In general, antibodies raised against the progesterone-7α-carboxyethylthioether antigen did not show better specificity than those raised against the 11α-hydroxyprogesterone-hemisuccinate antigen, regardless of whether they were tested in a homologous or in a heterologous system. This indicates that specificity could not be improved by site heterology (variation in the site of attachment of the hapten to the macromolecular carrier in the immunogen). Furthermore, when antibody 4XVIII was incubated with the heterologous tracer P-7-HRP, it appeared that the degree of cross-reactivity with nearly all steroids tested increased compared to incubation with the homologous tracer (P-11-HRP). This effect of heterology is in agreement with previous observations reported by Bosch and Schuurs (7). It was surprising, however, that binding did indeed occur. In fact, binding was not expected at all, because the sites of attachment of progesterone on the antigen (11-position) and on the tracer (7-position) were just opposite to each other, which theoretically might lead to maximum steric hindrance. All other antibodies did only bind to the homologous or nearby homologous tracer.

Association constants (K_A) of all monoclonal antibodies for their homologous tracers were rather low. However, when tested with the heterologous

TABLE IA
Characteristics of monoclonal antibodies raised against 11α-hydroxyprogesterone-hemisuccinate conjugated to bovine serum albumin (P-11α-BSA).

	Cross-reaction (%)						
	Vb7	Vb8	2Va	4XVIII	3XIII	1ab4	4XVIII
Tracer (P-6/7/11-HRP)	11	11	11	11	11	11	7
Steroid							
progesterone	100	100	100	100	100	100	100
11α-hydroxyprogesterone	34	60	215	37	75	321	71
11β-hydroxyprogesterone	7.1	11	32	1.7	15	24194	7.4
16α-hydroxyprogesterone	2.0	2.8	5.4	1.3	7.8	7.9	4.9
17α-hydroxyprogesterone	4.4	4.0	1.0	0.2	11	68	0.5
20α-hydroxyprogesterone	0.3	0.4	0.01	< 0.01	0.1	< 0.01	0.1
20β-hydroxyprogesterone	0.5	0.5	0.1	0.08	1.9	5.3	0.2
5α-pregnane-3,20-dione	26	19	409	11	7.2	< 0.01	75
pregnanedione	174	113	29	2.2	545	308	9.7
pregnenolone	3.7	3.5	12	0.08	2.3	< 0.01	0.5
17α-hydroxypregnenolone	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
5β-pregnane-3α,20β-diol	0.08	0.06	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
pregnanetriol	0.06	0.06	0.3	< 0.01	1.5	118	0.03
11-ketoprogesterone	28	45	214	10	230	3214	32
5-pregnene-3β,20α-diol	122	132	30	4.1	210	222	5.3
5β-pregnane-3α,17α,21-triol-20-one	0.8	0.7	1.0	0.08	1.3	< 0.01	0.4
1,4-diene-7α,21-diol-3,20-dione	0.4	0.2	< 0.01	0.3	0.06	15	0.5
ethiocholanolone	71	68	0.6	1.5	123	< 0.01	12
etiocholan-3,17-dione	9.8	14	0.4	1.8	26	18	13
cholesterol	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.01
cortisol	0.2	0.1	0.07	< 0.01	0.08	79	0.09
cortisone	0.6	0.5	< 0.01	< 0.01	< 0.01	40	0.3
corticosterone	1.5	0.2	0.2	< 0.01	0.5	727	0.1
desoxycorticosterone	2.8	2.9	0.3	0.3	2.2	323	0.6
aldosterone	0.1	0.07	0.2	< 0.01	< 0.01	108	0.03
testosterone	1.4	1.5	0.8	0.1	1.1	153	0.1
5α-dihydrotestosterone	1.2	1.8	0.2	0.09	1.1	4.5	0.2
5β-dihydrotestosterone	5.4	3.5	0.2	1.5	1.6	30	3.4
epitestosterone	0.5	0.3	< 0.01	0.07	0.07	8.1	1.8
17β-oestradiol	< 0.01	0.06	< 0.01	< 0.01	< 0.01	< 0.01	0.02
oestrone	0.05	0.3	< 0.01	0.01	< 0.01	< 0.01	0.06
oestriol	< 0.01	0.05	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
androsterone	4.7	5.9	2.6	0.2	6.7	< 0.01	0.4
androstenedione	0.9	3.3	0.09	0.2	7.2	13	0.7
androstenone	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Affinity constant (10^9 l/mol)	0.2	0.05	0.8	0.2	0.1	0.02	20
Immunoglobulin class	IgG_1	IgG_1	IgG_1	IgM	IgG_1	IgM	IgM

TABLE IB

Characteristics of monoclonal antibodies raised against progesterone-7α-carboxyethylthioether conjugated to bovine thyroid globulin (P-7α-BTG).

	Cross-reaction (%)							
	1XXI	2VIII	5XI	7II	7IV	5XVI	1XXIV	8V
Tracer (P-6/7/11-HRP)	6	6	6	6	6	7	7	7
Steroid								
progesterone	100	100	100	100	100	100	100	100
11α-hydroxyprogesterone	55	< 0.01	< 0.01	0.5	1.5	2.5	1.2	45
11β-hydroxyprogesterone	0.4	0.9	179	71	2.8	2.2	< 0.01	14
16α-hydroxyprogesterone	5.9	0.9	180	57	64	42	46	155
17α-hydroxyprogesterone	500	< 0.01	< 0.01	4.8	12	13	13	320
20α-hydroxyprogesterone	0.3	0.3	11	19	392	382	64	6500
20β-hydroxyprogesterone	1.8	6.2	65	50	27	17	5.3	23
5α-pregnane-3,20-dione	333	< 0.01	1.9	< 0.01	17	32	262	100
pregnanedione	0.6	5.0	250	400	4.0	2.0	8.1	16
pregnenolone	62	< 0.01	< 0.01	< 0.01	6.7	3.8	0.1	< 0.01
17α-hydroxypregnenolone	14	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
5β-pregnane-3α,20β-diol	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
pregnanetriol	0.7	13	3.2	37	< 0.01	< 0.01	0.1	15
11-ketoprogesterone	24	5.5	60	400	5.7	1.3	4.6	54
5-pregnene-3β,20α-diol	4.0	22	152	267	9.2	8.2	11	9.7
5β-pregnane-3α,17α, 21-triol-20-one	0.7	< 0.01	< 0.01	< 0.01	0.5	1.6	0.6	0.7
1,4-diene-7α,21-diol- 3,20-dione	0.1	< 0.01	< 0.01	< 0.01	5.4	4.7	4.3	0.2
ethiocholanolone	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.04	< 0.01	< 0.01
etiocholan-3,17-dione	0.7	0.28	28	18	2.7	1.0	2.6	2.3
cholesterol	7.8	< 0.01	< 0.01	< 0.01	< 0.01	0.08	0.08	< 0.01
cortisol	0.2	< 0.01	< 0.01	< 0.01	< 0.01	0.02	0.06	0.1
cortisone	0.07	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.09	< 0.01
corticosterone	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.2	0.2	< 0.01
desoxycorticosterone	2.1	0.3	1.9	5.1	73	57	258	3.2
aldosterone	< 0.01	< 0.01	< 0.01	1.9	< 0.01	< 0.01	0.6	< 0.01
testosterone	7.0	23	44	69	8.5	10	31	5.4
5α-dihydrotestosterone	3.9	5.5	0.6	14	0.8	0.3	41	12.1
5β-dihydrotestosterone	0.4	1.5	3.3	3.0	0.9	1.9	3.2	< 0.01
epitestosterone	< 0.01	< 0.01	< 0.01	4.0	3.9	5.7	13	0.05
17β-oestradiol	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
oestrone	< 0.01	< 0.01	< 0.01	< 0.01	0.1	< 0.01	0.08	< 0.01
oestriol	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
androsterone	0.1	< 0.01	< 0.01	< 0.01	0.4	0.4	4.0	0.5
androstenedione	< 0.01	< 0.01	< 0.01	< 0.01	27	25	104	1.0
androstenone	< 0.01	< 0.01	< 0.01	< 0.01	1.1	0.04	1.0	< 0.01
Affinity constant (10^9 l/mol)	0.2	0.5	0.01	0.01	0.6	0.6	0.03	0.03
Immunoglobulin class	IgG_1	IgG_{2b}	IgG_{2b}	IgG_{2b}	IgG_{2b}	IgG_{2a}	IgG_{2b}	IgG_{2a}

tracer (P-7-HRP) the affinity for progesterone of antibody 4XVIII increased from 2 x 10^8 l/mol to 2 x 10^{10} l/mol. Using P-7-HRP as a tracer, antibody 4XVIII showed both high affinity and high specificity. For the other antibodies there was no clear cut relationship between specificity and affinity.

The antibodies produced were mostly of the IgG_1 and IgG_{2b} class (Table 1). Immunization with P-11α-BSA tended to give rise to IgG_1 and IgM monoclonal antibodies (for instance antibodies of cell line 4XVIII appeared to be of IgM class) and immunization with P-7α-BTG to IgG_{2a} and IgG_{2b} antibodies. Affinity and specificity did not appear to be related to a particular class of immunoglobulin.

Sensitivity of the EIA's utilizing the monoclonal antibodies was defined

TABLE 2

Sensitivity of anti-progesterone monoclonal antibodies.

	Tracer (P-6/7/11-HRP)	$\frac{1}{2}B_{max}$ (pg)	Detection limit (pg)
Hybridomas to P-11α-BSA			
Vb7	11	2,300	200
Vb8	11	3,000	400
2Va	11	300	20
4XVIII	11	1,250	20
3XIII	11	3,300	200
lab4	11	60,000	4,000
4XVIII	7	18	1
Hybridomas to P-7α-BTG			
1XXI	6	2,000	50
2VIII	6	1,800	800
5XI	6	60,000	5,000
7II	6	120,000	10,000
7IV	6	900	50
5XVI	7	600	60
1XXIV	7	5,500	600
8V	7	10,000	1,000

as 1) the smallest amount of progesterone detectable at the 95% confidence limit (detection limit) and 2) the concentration of progesterone causing a 50% reduction of maximum binding in the standard curve ($\frac{1}{2}B_{max}$), and is shown in Table 2. As expected from affinity constants, sensitivity of the antibodies was rather low when P-7-HRP was used as a tracer, except for antibody 4XVIII. In contrast to assays with polyclonal antibodies (1,8), sensitivity could not be improved by introducing a heterologous (P-6-HRP) tracer in the assays with monoclonal antibodies against the 7α-antigen. This also contrasts with the improved sensitivity obtained with the antibody against the 11α-antigen (4XVIII) incubated with P-7-HRP, as compared to incubation with P-11-HRP. Ascites fluid containing antibody 4XVIII had the same binding properties as antibodies in the cell culture medium. The titer of specific antibody in the ascites fluid was 1:13,500,000, and in culture medium 1:12,000.

Because of the relatively high titer, the high sensitivity, the high affinity for progesterone and the relatively low cross-reactivity with other steroids, monoclonal antibody of hybridoma cell line 4XVIII was considered to be useful for standardizing the enzyme immunoassay of milk-progesterone.

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CHAPTER 3

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST THE H-Y ANTIGEN

P. Booman[a], L. Kruijt[a], M. Tieman[a], J.A. Piedrahita[b], R. Veerhuis[a], P. de Boer[c] and F.E. Ruch[d]

[a]Research Institute for Animal Production "Schoonoord", P.O. Box 501, 3700 AM Zeist (The Netherlands)
[b]Department of Animal Science, University of California, Davis, CA 95616 (U.S.A.)
[c]Department of Genetics, Wageningen Agricultural University, Gen. Foulkesweg 53, 6703 BM Wageningen (The Netherlands)
[d]ImmuCell Corporation, 966 Riverside Street, Portland, MA 04103 (U.S.A.)

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Abstract

Monoclonal antibodies against the H-Y antigen were produced using spleen cells from female C57BL/6 mice hyperimmunized with cells from syngeneic males. Anti-H-Y-positive clones were detected by enzyme immunoassays. Supernatant fluids from Daudi cell cultures and testicular cell preparations taken from mice, rabbits or calves served as presumptive sources of H-Y antigen. In addition, testis supernatant from genetically sterile mice was used. Male specificity was ascertained by the fact that the antibodies could be absorbed with spleen cells from male but not from female mice. Binding of the antibodies to H-Y antigen on the surface of male and female cells, obtained from a number of tissues and species, was confirmed by an indirect immunofluorescence assay. Several monoclonal antibodies appeared to be positive in all assays tested, suggesting that the molecule conferring the H-Y antigenicity lacks species-specificity and appears to be identical for soluble and membrane-bound H-Y antigen.

Introduction

The H-Y antigen is a minor histocompatibility antigen that is serologically detectable on the surface of male mammalian cells. It was first recognized in an inbred strain of mice in which the females rejected male, but not female, skin grafts (Eichwald and Silmser, 1955). Goldberg et al. (1971) later demonstrated that tissue rejection was accompanied by antibody formation to the presumptive H-Y antigen. The anti-H-Y antisera could identify cells from the heterogametic sex in serological systems. Also, murine anti-H-Y antisera were shown to cross-react with H-Y on cells from numerous species ranging from fish to man (Wachtel, 1983a).

There has been some controversy as to whether there are two different male-specific antigens, one detected by transplant rejection (the H-Y antigen) and one detected by serological methods (serologically detectable male antigen or SDM, Simpson et al., 1982). However, the results of Koo and Varano (1981) and Koo et al. (1983) contradicted those of Simpson et al. (1982). In addition, Wachtel et al. (1984) reported that the H-Y transplantation and SDM antigens were either identical or highly cross-reactive. In this paper, therefore, the term H-Y antigen will be used with the under-

standing that there is still controversy as to whether SDM and H-Y antigen are identical molecules.

Because of the exclusive presence of H-Y antigen in the mammalian male (Wachtel et al., 1975) and its detection early in embryonic development (Krco and Goldberg, 1976), it has become possible to predict the phenotypic sex of the offspring on the basis of embryonic H-Y antigen expression (for reviews, see Booman, 1986; Anderson, 1987). Most experiments designed to determine the sex of embryos have been performed with polyclonal antisera obtained by hyperimmunizing female mice or rats with syngeneic male spleen cells. Although such systems can be accurate (White et al., 1982, 1984), the use of polyclonal antisera may give rise to a relatively high degree of non-specific background (Piedrahita and Anderson, 1985), probably caused by contamination with autoantibodies (Koo et al., 1973). Furthermore, only a low percentage of mice have a good antibody response to H-Y antigen and their sera are usually low titered and of low affinity. The availability of monoclonal anti-H-Y antibodies may resolve some of these difficulties, thereby enabling the production of a standardized assay for identification of the sex of preimplantation embryos from economically important species. We report here the production and characterization of monoclonal antibodies against the H-Y antigen which were found to be male-specific on the basis of their binding specificity in a variety of tests.

Materials and Methods

Production of hybridomas

Hybridoma cell lines were produced by standard techniques. Female C57BL/6 (B6) mice (Harlan Olac) received multiple intraperitoneal injections of B6 male spleen cells (5×10^7/dose) over a period of 9 weeks. Three to four days before fusion, the animals were boosted intravenously with 2.5×10^7 male spleen cells. In total, five fusions between X63-Ag8.653 myeloma cells and spleen cells from the immunized B6 female mice were performed as previously described (Booman et al., 1984).

Detection of anti-H-Y antibody production

Hybridoma supernatants were first screened for the presence of anti-H-Y antibodies in enzyme immunoassays (EIAs) based on the reaction of secreted antibodies and soluble H-Y antigens. Soluble H-Y antigen has been detected in a.o. the supernatant fluid of Daudi cell cultures (Nagai et al., 1979) and in testicular cell preparations (Müller et al., 1978). We, therefore, used testis supernatant (TS) from homogenates of B6 testes prepared according to Wachtel et al. (1980) and Daudi supernatant (DS) from Daudi cells cultured according to Nagai et al. (1979).

Microtiter plate wells were coated with serial dilutions of TS or DS, blocked with 2% normal serum from female rabbits and incubated with hybridoma supernatants, diluted 1/10 in buffer containing 1% rabbit serum, for 3 h at room temperature (rT; 21°C). Rabbit anti-mouse Ig antiserum conjugated to horse-radish peroxidase (RAM-Ig-HRP; Nordic Immunological Laboratories) was then added and the plates were incubated for 2 h in the dark. After washing with 0.05% Tween 80, a substrate solution containing tetramethylbenzidine (Fluka) and hydrogen peroxide was introduced. After an incubation period of 45 min the reaction was stopped by adding 4N sulfuric acid and the absorbance was measured spectrophotometrically at 450 nm (Titertek multiskan). Mouse serum containing polyclonal antibodies against the H-Y antigen was used to validate the assays.

Characterization of anti-H-Y antibodies

Anti-H-Y antibody-producing clones detecting mouse testicular and Daudi H-Y antigens were further tested for their cross-reactivity to soluble H-Y antigen from rabbit and calf testes. Preparation of testicular supernatants and monoclonal binding assays were performed as described for mouse H-Y antigen.

Additional screening assays were done on TS from genetically sterile mice. Two male-sterile karyotypes were used: T(1;13)70H/T(1;13)1Wa (De Boer et al., 1986) and Is(7;1)40H/+ (Searle et al., 1983). The former type was bred by one of us (P. de B.) and identified by peripheral blood culture karyotyping and by two sterile matings. The latter was kindly supplied by dr. A.G. Searle, Medical Research Council Radiobiology Unit, Chilton, Oxon, U.K.

Specificity of the EIAs for a male-specific antigen was ascertained by serological absorption. Antibody-containing supernatants of positive clones were absorbed with spleen cells obtained from male or female B6 mice and then scored for residual activity in the DS and TS EIAs. For absorption, aliquots of supernatants (50 μl) were diluted 1/4 and incubated at 4°C for 1 h with 2.5×10^5, 5×10^5, 7.5×10^5 or 1×10^6 splenocytes. Subsequently, the cells were discarded and the supernatants diluted to 1/10, 1/25 and 1/50 for use in the EIAs. As a control, supernatants were treated similarly but in the absence of cells.

In order to evaluate the ability of the anti-H-Y antibodies to bind to H-Y antigen present on cell surfaces, supernatants of positive clones were tested in indirect immunofluorescence assays on both male and female cells. Cell suspensions were prepared from different murine, rat and porcine tissues. In the first series of tests, rat and porcine Sertoli cells were isolated and cultured according to the procedure of Tung and Fritz (1977). After 4-7 days of culture, cells were incubated with diluted supernatants of anti-H-Y clones for 2 h at rT and washed by centrifugation. Subsequently, FITC-conjugated rabbit anti-mouse Ig antiserum (RAM-Ig-FITC; Nordic Immunological Laboratories) was added for 1 h at rT in the dark. Unbound secondary antibodies were removed by washing and centrifugation and the cells were examined under an inverted fluorescence microscope. As a negative control, culture supernatant of a rabies-specific monoclonal antibody was used. Non-specific binding to female cells was tested on primary cell cultures of rat ovary cells.

As suitably matched counterparts, primary cell cultures of male and female mouse fibroblasts were compared in reactivity with anti-H-Y antibodies. Male but not female fibroblasts have been found to be positive for H-Y antigen (Koo et al., 1981; Müller and Bross, 1979). Fibroblasts were isolated and cultured according to procedures described by Koo et al. (1981).

To determine the level of immunoglobulin production and the immunoglobulin (sub)classes of the anti-H-Y antigen clones, microtiter plates were coated with RAM-Ig and specific goat anti-mouse (sub)class Ig (Nordic Immunological Laboratories), respectively. The assays were then performed as described for the DS and TS EIAs.

Results

Binding of antibodies with Daudi and testis supernatants

After performing five fusions, we obtained a total of ten hybridoma cell lines secreting monoclonal antibodies that reacted with DS in an EIA (Table 1). When tested on TS from normal mice, antibodies with low affinity for soluble H-Y antigen from Daudi cells were either negative or weakly positive, while the high affinity antibodies, as determined by binding to DS, reacted strongly with TS (Table 1). No antibodies positive in the TS-based EIA and negative in the DS-based EIA were found.

From Table 1 it can be seen that binding of antibodies to TS from rabbits, calves and genetically sterile mice was in the same range as binding to DS and TS from normal mice. Although antibodies were raised against mouse H-Y antigen, binding of the monoclonal antibodies to rabbit and calf TS demonstrates high cross-reactivity with rabbit and bovine H-Y antigen. Cross-reactivity with human H-Y antigen can be concluded from binding to DS. With one exception, antibodies gave highest binding to TS from Is40H/+ sterile mice followed by TS from T70H/T1Wa mice and rabbits.

Protein concentrations of supernatant fluids used for coating varied significantly, not only between DS and all TSs, but also between the different TSs (Table 2). Compared with DS, TS is a crude antigen source. The amount of TS required for optimal binding of monoclonal antibody 5 XI in the EIA (64 or 32 μg/ml) was large in comparison with the amount of DS (10 μg/ml). Although the total protein content from rabbit, and particularly from bovine, TS was much higher than from the TS of normal mice, the proportion of H-Y antigen to total protein is constant. Evidently, in both groups of sterile mice, H-Y antigen represents a larger portion of the total protein secreted in the testis than in normal mice. On a total protein basis two times less TS from sterile than from normal mice was required for coating. Just as with bovine TS, the high total protein concentration of TS from the T70H/T1Wa sterile mice did not influence the relative amount of H-Y antigen present.

TABLE 1

Reactivity of anti-H-Y monoclonal antibodies in different assays.

	Cell lines									
	2 IX	4 VII	8 XX	1 III	2 XXIII	2 XXIV	5 XI	5 X	8 IV	HY-1
O.D. in EIAs[a] *based on:*										
Supernatant Daudi cells	112	1420	242	642	377	272	1332	288	295	793
Supernatant testis homogenates										
Normal mice	-12	1332	3	376	280	-12	994	52	-10	570
Rabbits	-91	1716	-78	333	464	-81	1180	28	5	520
Calves	-52	1100	-66	212	149	-130	952	-48	-118	275
Is40H/+ sterile mice	- 6	1726	46	552	374	-17	1509	130	76	876
T70H/T1Wa sterile mice	24	1400	14	446	406	15	1112	91	35	582
Serological absorption[b]	-	++	±	+	+	-	++	+	±	++
Immunofluorescence[c] *on:*										
Porcine Sertoli cells	$\pm^{3}$	$++^{1}$	$\pm^{3}$	$++^{2}$	$+^{2}$	$\pm^{3}$	$+++^{1}$	$++^{3}$	$\pm^{3}$	$++^{1}$
Rat Sertoli cells	-	$+^{1}$	-	$++^{2}$	$\pm^{3}$	$\pm^{3}$	$++^{1}$	$+++^{3}$	$\pm^{3}$	$++^{1}$
Rat ovary cells	-	$\pm^{3}$	-	$+^{3}$	-	-	$\pm^{3}$	$++^{3}$	-	$\pm^{3}$
Mouse male fibroblasts	$\pm^{3}$	$+^{1}$	$\pm^{3}$	$+^{2}$	$+^{2}$	$\pm^{3}$	$++^{1}$	$++^{3}$	$\pm^{3}$	$+^{1}$
Mouse female fibroblasts	-	$\pm^{3}$	-	$+^{3}$	-	-	$\pm^{3}$	$++^{3}$	-	$\pm^{3}$
Ig titer[d]	900	200	200	600	500	800	300	1500	300	300
Immunoglobulin (sub)class	IgG_{2b}	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM

[a]The results of the EIAs in which the binding of monoclonal antibodies (1:10 dilution) to various H-Y antigen sources was assessed, are expressed as absorbance (x 1000) at 450 nm, corrected for blank values. Values obtained with class- and concentration-matched anti-rabies monoclonal antibodies did not differ significantly from blank values.

[b]Capacity of male C57BL/6 spleen cells to absorb the H-Y binding potential, as measured in the EIAs. As a control, monoclonal antibodies were absorbed with spleen cells from female C57BL/6.

[c]Binding of monoclonal antibodies (1:5 dilution) to various cell cultures in an indirect fluorescence assay. Fluorescence intensity: +++ strong; ++ relatively strong; + intermediate; ± weak; - no fluorescence. Fluorescence patterns : 1, linear; 2, linear as well as diffuse background; 3, homogeneous background only.

[d]Ig titers were determined in a double antibody solid phase EIA employing (sub)class specific antibodies. Titers are expressed as $\frac{1}{2}B_{max}$ $(x\ 10^{-1})$.

TABLE 2

Protein content of various supernatant fluids and concentration required for optimal binding of monoclonal antibody 5 XI to H-Y antigen in these fluids.

	Total protein[a] (mg/ml)	Protein concentration (μg/ml) used for coating[b]
Supernatant Daudi cells	0.01	10
Supernatant testis		
Normal mice	7.18	64
Rabbits	11.99	64
Calves	35.42	64
Is40H/+ sterile mice	6.84	32
T70H/T1Wa sterile mice	16.75	32

[a]Determined by standard Coomassie Blue assay for total protein in a sample with Biorad Protein Assay Kit; after Bradford (1976).
[b]Absorbance of 0.9 at 450 nm with 1:10 diluted supernatant of cell line 5 XI.

Absorption

Confirmation of male specificity by absorption revealed that, in general, absorption with male spleen cells caused a fall in residual activity of the antibodies with both DS and TS. Loss of reactivity after absorption with female cells was not more than what could be attributed to non-specific absorption. Absorption with small numbers of cells caused a slight but consistent difference between male-absorbed and female-absorbed antibody. A representative test using supernatant from hybridoma cell line 5 XI is illustrated in Fig. 1. A progressive fall in residual activity of the antibodies was observed with increasing numbers of male spleen cells. When a large number of cells were used for absorption, however, results were not as consistent.

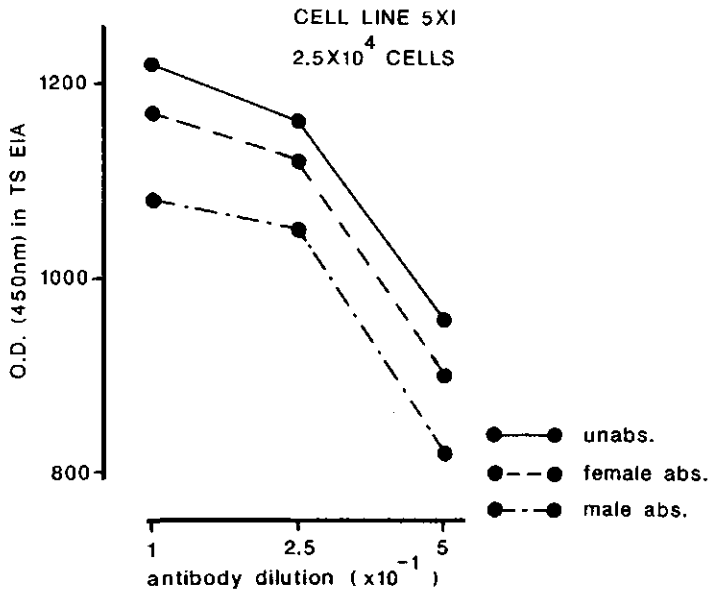

Fig. 1. Reaction of monoclonal antibody 5 XI to mouse TS in the EIA after absorption with spleen cells from female or male B6 mice.

Binding of antibodies to cell-surface H-Y antigen

Characteristic fluorescence patterns of antibodies directed against cell-surface antigens were observed in rat and porcine Sertoli cells by indirect fluorescence assays. Antibodies 4 VII, 1 III, 5 XI and HY-1, which were clearly positive in the EIAs, showed weak lines of fluorescence along the cell surface of 15-30% of the cells (Table 1). Some non-specific binding to ovary cells was observed with all the three monoclonal antibodies showing highest anti-H-Y activity in the EIAs (4 VII, 5 XI and HY-1). Non-specific binding was characterized by homogeneous background fluorescence with no obvious cell-surface fluorescence. Antibodies of cell lines 5 X and 1 III showed a relatively high background. In experiments on male and female mouse fibroblasts, both specific and non-specific patterns of fluorescence were similar to those seen on Sertoli and ovary cells, respectively. The intensity of fluorescence, however, was lower in fibroblasts than in Sertoli cells.

Discussion

The weak immunogenic character of the H-Y antigen is demonstrated by the low number of hybridoma cell lines secreting anti-H-Y antibodies. The limited number of clones obtained in five fusion experiments also confirms the generally reported poor polyclonal antibody response after hyperimmunization of female mice with male spleen cells. All but one of the monoclonal antibodies are of the IgM class, which is according to expectation, since the H-Y antigen is a glycoprotein, the serological determinant most likely being a carbohydrate chain with a galactose terminal residue (Shapiro and Erickson, 1981). Polysaccharides alone predominantly elicit IgM antibodies (see e.g., Kannagi and Hakomori, 1986), with generally low affinity (Klein, 1982). In addition, it has been reported that hyperimmunization may also cause the forming of IgM antibodies.

It has been shown previously that monoclonal antibodies against the H-Y antigen bind to soluble H-Y antigen in both DS and mouse TS (Brunner et al., 1984). For optimal binding, Brunner et al. (1984) coated microtiter plates with 5 μg/ml DS and 44 μg/ml TS, compared with 10 μg/ml DS and 64 μg/ml TS in our experiments (Table 2). The total protein content of our TS from normal mice was found to be higher than that reported by Brunner et al. (1984) (7.18 vs. 4.40 mg/ml). This could be due to variability in the procedure for TS preparation as well as the sexual status of the mice. Compared with DS, the H-Y antigen represents only a small portion of the total protein secreted in the testis of normal mice. In both lines of sterile mice, the proportion of H-Y antigen to total protein appears to be higher. Sperm production was absent in both mutants, but activity of Sertoli cells was normal. Apparently, the absence of spermatozoa, which normally absorb H-Y antigen (Ohno, 1982), increased the relative amount of free H-Y antigen. Our data also confirm earlier findings that the H-Y antigen is secreted by Sertoli cells (Müller et al., 1978; Zenzes et al., 1978) and agree with Brunner et al. (1984) who found that the supernatant of an established mouse Sertoli cell line contains the H-Y antigen. The higher protein content of the T70H/T1Wa rete testis fluid compared with Is40H/+ and control mice could be due to the fact that cell death probably occupies stages in-between mid-pachytene and round plus elongating spermatids (De Boer et al., 1986). Thus, before Sertoli cells phagocytose, the products of cell degeneration could have added to the high protein

concentration in rete testis fluid. Apparently, this is not the case with Is40H/+ where cell degeneration uniformly takes place at stage IV of the cycle of the seminiferous epithelium (Searle et al., 1983).

When comparable amounts of protein of TS from rabbits, calves and normal mice are coated to microtiter plates, similar rates of binding of the monoclonal antibodies are obtained, indicating high cross-reactivity of the antibodies with H-Y antigen from different species. These data confirm previously reported experiments showing that the H-Y antigen of vertebrates and non-vertebrates is either identical or highly cross-reactive (see review by Wachtel, 1983a).

With regard to the use of bovine TS as the solid phase antigen, it can be concluded that the total protein content from bovine TS is higher than TS from rabbits and (sterile) mice. However, the proportion of H-Y antigen to total protein appears to be constant. As suggested by Bradley et al. (1987), bovine TS constitutes an easily obtainable source of large amounts of antigenic protein(s) for use in the EIA.

The similarity in degree of binding to DS/TS, the reduced activity after absorption of the monoclonal antibodies with male cells, and the male-specific fluorescence on intact viable cells demonstrate that the serologically detectable determinants of cell-surface and soluble H-Y antigen are either identical or highly cross-reactive. The binding to cell-surface H-Y antigen was expected since antibodies were obtained by immunizing female mice with intact male cells. The slight non-specific binding of antibodies from cell lines 4 VII, 5 XI and HY-1 with female cells is remarkable because of consistent high anti-H-Y activity in all assays (Table 1). However, if the monoclonal antibodies are directed to carbohydrate moieties, as might be the case according to Shapiro and Erickson (1981), slight cross-reactivity with another glycosylated protein present on the surface of both female and male cells could be an explanation for our results. Also, Wachtel (1983b) suggested that H-Y antibodies may be MHC-restricted and may therefore recognize H-Y antigen in association with MHC-determined antigens, which could result in some non-male-specific binding to female cells. Another cause of the non-specific binding might be limited expression of H-Y antigen on female cells (Zenzes and Reed, 1984).

With regard to the choice of type of cells taken for demonstration of cell-surface H-Y antigen, Sertoli cells have the advantage that they are assumed to have a high level of expression of H-Y antigen. Indeed, with

some antibodies (4 VII, 1 III, 5 XI and HY-1), fluorescent lining of the cells, a characteristic pattern of cell-surface fluorescence, was observed. However, that does not prove male specificity because control female counterparts to Sertoli cells are lacking. Using fibroblasts, a similar but weaker fluorescence pattern on male cells was observed, indicating weaker expression of the H-Y antigen.

Finally, the amount of mouse Ig in supernatants of hybridoma cell cultures correlates positively with the results of the DS and TS EIAs, reflecting the specificity of the monoclonal antibodies for H-Y antigen. For instance, the most promising antibodies (4 VII, 5 XI and HY-1) were low titered in the EIA for mouse Ig and less promising antibodies (e.g. 2 IX, 2 XXIV and 5 X) were high titered, indicating high and low affinity for H-Y antigen, respectively.

In a recent study (Booman et al., 1989) the most promising monoclonal antibodies, 4 VII, 5 XI and HY-1, have been evaluated for their efficiency in sexing Day 7 bovine preimplantation embryos.

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CHAPTER 4

SEXING BOVINE EMBRYOS WITH MONOCLONAL ANTIBODIES AGAINST THE H-Y ANTIGEN

P. Booman[a], L. Kruijt[a], R. Veerhuis[a], A.M. Hengst[a], M. Tieman[a] and F.E. Ruch[b]

[a]Research Institute for Animal Production "Schoonoord", P.O. Box 501, 3700 AM Zeist (The Netherlands)
[b]ImmuCell Corporation, 966 Riverside Street, Portland, MA 04103 (U.S.A.)

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Abstract

Monoclonal antibodies against the H-Y antigen have been used to identify the sex of preimplantation bovine embryos. In an indirect immunofluorescence assay no clearcut discrimination between positive (male) and negative (female) fluorescent embryos could be obtained, mainly because of non-specific binding of the monoclonal antibodies to the embryonic cells. However, after modifying the technique in order to reduce non-specific binding, the number of false positives after sexing under these conditions was greatly reduced, which suggests that the monoclonal antibodies detect a male-specific antigen. Although the H-Y antigen was not detected on about half of the male embryos, the experiments indicated that it is possible to identify the sex of bovine embryos based on detection of H-Y antigen with monoclonal antibodies.

Introduction

It is likely that a successful technique for the determination of the sex of embryos prior to implantation will be used widely in dairy cattle breeding and also will contribute to extension of the use of multiple ovulation and embryo transfer at the commercial level (Booman, 1988). The development of several invasive as well as non-invasive techniques for sexing bovine embryos has been reported. Invasive techniques include cytological methods (King, 1984) and chromosome hybridization of Y-chromosome-specific labeled c-DNA (Leonard et al., 1987). Invasive techniques have several disadvantages, such as possible reduced embryo viability owing to damage of embryonic cells. Furthermore, embryos cannot be cleaved after such treatment. Therefore, non-invasive methods for sexing embryos are preferable. Quantification of differences in metabolic activity of X-linked enzymes in male and female embryos, based on the assumption that in Day 7 bovine embryos X-chromosome inactivation has not yet taken place, has been proposed as a non-invasive method (Rieger, 1984). Data from experiments designed to test this hypothesis in the mouse have, however, not been encouraging (Williams, 1986). On the other hand, immunological detection of a male-specific protein, referred to as either the H-Y antigen or serologically detectable male antigen, has shown to be a relatively

successful non-invasive method of identifying the sex of embryos (for reviews, see Booman, 1986 and Anderson, 1987).

As far as we are aware, nearly all experiments to identify the sex of embryos based on the detection of the H-Y antigen have been performed with polyclonal antibodies generated by hyperimmunizing female mice or rats with syngeneic male spleen cells. Monoclonal antibodies, that offer a big advantage due to their unlimited availability and their specificity, were used in only two studies: White et al. (1983) sexed mouse embryos and Wachtel (1984) reported preliminary work on sexing bovine embryos with a monoclonal antibody.

Recently we described the development of monoclonal antibodies to the H-Y antigen (Booman et al., 1989). The objective of the present study was to determine the discriminating capacity of some of these monoclonal antibodies to sex bovine embryos when used in an indirect immunofluorescence assay. Although a discrimination between male and female embryos could be made, evaluation of the staining patterns was fairly subjective because of non-specific binding of the monoclonal antibodies to the embryonic cells. Therefore, a series of experiments with mouse embryos was performed in order to try to increase the ratio of specific to non-specific binding. We optimized procedures for washing away unbound antibodies, and tried to establish whether IgG-like fragments of the IgM monoclonal antibodies could more easily penetrate the zona pellucida. Varying concentrations of pronase were used to make the zona pellucida more porous. In order to increase the fluorescent signal after binding of the anti-H-Y antibody to the H-Y antigen, a secondary antibody with a strongly fluorescent label was tested. In addition the phenomenon of modulation of the H-Y antigen was studied. Based on the results obtained from these experiments a revised test was set up. In the present paper the results of sexing bovine embryos under "standard" conditions and under "improved" conditions are compared.

Materials and Methods

Sexing bovine embryos under standard conditions

Antibody-producing cells of hybridoma cell line 5 XI (Booman et al., 1989) were cultured in serum-free medium (HB 102; Hana Media) and super-

natants concentrated tenfold by Amicon filtration (XM300), aliquoted and frozen at -80°C until use.

Bovine embryos were recovered at slaughter from the uteri of superovulated donor Friesian cows 6-7 days after estrus (Day 0=first day of estrus). Non-transferable embryos according to the embryo qualification system of Lindner and Wright (1983) were excluded from this experiment.

Embryos were transferred to a Petri dish containing 1 ml of anti-H-Y monoclonal antibody culture supernatant, which had been diluted 1:3 in Whitten's medium (WM) pH 7.2 (Whitten and Biggers, 1968) supplemented with 10% IgG-free heat-inactivated fetal calf serum (FCS) (Gibco), and incubated for 90 min. After two 1-min washes followed by a 15-min wash in WM, embryos were incubated for 60 min in the dark in 3 ml goat anti-mouse IgM-(Fc)-FITC (GAM-FITC) (Nordic Immunological Laboratories) secondary antibody diluted 1:200 in WM + FCS. After washing, each embryo was transferred to one well of a 24-well plate, containing 2 ml WM + FCS. Fluorescence was evaluated by use of an inverted phase contrast microscope (IMT2 Olympus) at 400x magnification. Embryos were classified as either fluorescent (H-Y positive) or non-fluorescent (H-Y negative). All procedures were carried out at 22°C. Staining patterns were recorded using a highly sensitive SIT video camera (Grundig electronic, SN76).

Following classification, colchicine (Sigma) at a final concentration of 0.5 $\mu g\ ml^{-1}$ was added to each well and after culturing for 3 h at 37°C, embryos were karyotyped as described by Dyban (1983). Only 100% readable karyograms were taken into account.

Reduction of non-specific binding and magnification of positive signal

Concentrated serum-free supernatant of cell line 5 XI was used for all experiments on mouse embryos, with the exception of the fragmentation experiments. For preparation of IgG-like fragments, IgM was isolated from ascites fluid of respectively cell lines 5 XI, 4 VII and HY-1 (Booman et al., 1989) by subsequent centrifugation, ammonium sulphate precipitation and gel filtration on ACA 22 (LKB). Fractions were assessed for the presence of IgM in an enzyme immunoassay (EIA) detecting mouse Ig (Booman et al., 1984). Fragmentation was essentially performed as described by Matthew and Reichardt (1982) with minor modifications.

Mouse embryos were flushed from the oviducts and uteri of mated C57BL/6

(Harlan Olac) female mice on the morning of Day 3 (Day 0=day of copulation plug). Basically, the incubation with antibody and the recording of fluorescence was as described for the bovine embryos.

Washing procedure

Groups of at least 20 mouse embryos were sexed according to the standard procedures on the understanding that extensive washing steps of respectively two 5-min washes followed by a 10-min wash and two 10-min washes followed by a 20-min wash were introduced after incubation with the anti-H-Y and secondary antibody. Fluorescence patterns were compared with the patterns of a group of embryos with the standard washing procedure of two 1-min washes and a single 15-min wash.

Accessability of embryonic cells

Embryos were treated with pronase (Hoechst) in Dulbecco's PBS without Ca^{2+}/Mg^{2+} at concentrations of 0.5, 0.25, 0.15, 0.10 and 0.05% during 5 or 10 min at 37°C in order to find the critical concentration of pronase at which the zona was no longer removed. Groups of at least 20 embryos were pretreated for various time periods with different concentrations below the critical concentration, and then sexed according to the standard procedures. Fluorescence patterns of pronase-treated embryos were compared with patterns of untreated ones.

Mouse embryos were sexed with a cocktail of fragmentated 5 XI, 4 VII and HY-1 consisting of 0.1 mg ml^{-1} of each fragment preparation. Except for the secondary antibody (rabbit anti-mouse Ig-FITC; Nordic Immunological Laboratories) other conditions were identical to the standard procedure. Fluorescence patterns were compared with patterns of embryos obtained after incubation with a mixture of the purified anti-H-Y antibodies before fragmentation at a concentration of 10 μg ml^{-1} each.

Modulation of the H-Y antigen

In order to study whether anti-H-Y monoclonal antibodies induce modulation of the H-Y antigen on the cell surface, groups of at least 20 embryos were sexed either at 4°C both with and without sodium azide (NaN_3) (0.1%) or at 22°C with NaN_3, and fluorescence was evaluated. As a control a group of embryos was sexed under standard conditions (22°C without NaN_3).

Amplification system

In order to amplify the positive signal resulting from binding of monoclonal anti-H-Y antibody to the H-Y antigen, goat anti-mouse IgM(Fc)-R-phycoerythrin (GAM-RPE) was compared to GAM-FITC on mouse embryos as a secondary antibody. RPE produces a signal at least six times stronger than FITC (Kronick and Grossman, 1983). RPE preparations were obtained from Biomeda Corp., Foster City, CA., as well as from Southern Biotechnology Associates Inc., Birmingham, AL.

In order to establish the effect of RPE on the viability of mouse embryos, RPE-treated embryos were transferred to the uteri of recipients (Balb/c) (Harlan CPB) on Day 2 of pseudopregnancy. Control transfers were performed with embryos that were cultured for 2-3 h in WM + FCS only.

Sexing bovine embryos under "improved" conditions

Bovine embryos of 6-7 days old were collected and then sexed with some modifications of the standard procedure. A cocktail of purified 5 XI, 4 VII and HY-1 was used for the first antibody step. Extensive washing procedures of two 10-min and a single 20-min wash were introduced and GAM-RPE was the secondary antibody. The whole procedure was performed at 4°C.

Results

Evaluation of sexing bovine embryos under standard conditions

Most bovine embryos flushed at Day 7 were at the early blastocyst stage. Three categories of (non-)fluorescent embryos were distinguished: H-Y-positive embryos, H-Y-negative embryos and an intermediate group of embryos (partly assigned as fluorescent or H-Y positive, partly as non-fluorescent or H-Y negative). The characteristics of the fluorescence patterns of these categories are described in Table 1.

In lower quality embryos (grades III and IV according to Lindner and Wright, 1983) plaques of non-specific fluorescence were often associated with dead material or with less viable cells. Fluorescence had to be evaluated on the basis of the viable part of the cell mass. Lower quality embryos led to a more subjective evaluation and required more experien-

TABLE 1

Characteristics of fluorescence patterns of bovine embryos sexed under standard conditions.

H-Y-positive embryos	H-Y-negative embryos	Intermediate category[a]
Patches of fluorescence over whole cell mass superimposed above background of equal fluorescence	Dark background No patches of fluorescence Occasionally few small bright spots or equal background of higher intensity without patches	Low intensity patches of fluorescence over whole cell mass or patches on part of cell mass

[a]Embryos with less clear staining characteristics; depending on intensity of fluorescence and size of fluorescent part of cell mass classified as fluorescent (H-Y positive) or non-fluorescent (H-Y negative).

TABLE 2

Numbers of embryos and accuracy of sex determination in each category of (non-)fluorescent bovine embryos sexed under standard conditions.

	No. of embryos observed		No. of readable karyotypes	No. of embryos sexed correctly		
	Fluorescent	Non-fluorescent		Fluorescent	Non-fluorescent	Total
H-Y-positive embryos	39(35%)	-	10	10/10(100%)	-	10/10(100%)
H-Y-negative embryos	-	44(39%)	12	-	12/12(100%)	12/12(100%)
Intermediate embryos	24(21%)	5(4%)	8	0/6 (0%)	1/2 (50%)	1/8 (13%)
Total	63(56%)	49(44%)	30(27%)	10/16(63%)	13/14(93%)	23/30(77%)

ce. The numbers of embryos with a fully readable karyogram in the different quality classes were too small, however, to calculate a correlation between misclassification and quality of the embryos.

A total of 112 bovine embryos were evaluated for expression of H-Y antigen; of these, 63 (56%) were classified fluorescent and 49 (44%) non-fluorescent. From 30 embryos a readable karyogram was obtained. Twenty-three embryos (77%) were assigned correctly: 10 out of 16 (63%) as H-Y-positive classified embryos and 13 out of 14 (93%) as H-Y-negative classified embryos. Detailed results are given in Table 2, from which it can be concluded that all misclassifications are in the relatively large intermediate group of embryos. The larger number of fluorescent as compared to non-fluorescent embryos seems to be a consequence of the false H-Y positives in this intermediate group of embryos.

Reduction of non-specific binding and magnification of positive signal

Sexing mouse embryos under standard conditions

Flushing of uteri of mice at Day 3 resulted mostly in embryos at the morula to early blastocyst stage. Sexing under standard conditions did not give a clearcut discrimination between H-Y-positive and H-Y-negative embryos. Staining ranged from highly fluorescent to non-fluorescent. Depending on the intensity of staining, embryos showing intermediate fluorescence were more or less subjectively assigned to the group of H-Y-positive embryos or to the group of H-Y-negative embryos. In Table 3 characteristic patterns of fluorescence are described for H-Y-positive and H-Y-negative mouse embryos when sexed under standard conditions.

Washing procedure

More extensive washing procedures resulted in a better discrimination between H-Y-positive and H-Y-negative embryos. In Table 4 changes in fluorescence characteristics for both positive and negative embryos are described after washing for 10, 10 and 20 min compared with characteristics after washing for 1, 1 and 15 min (standard procedure).

Accessibility of embryonic cells

Pretreatment of mouse embryos with 0.15% pronase for 5 min appeared to be the critical point for removal of the zona pellucida. Pretreatment

TABLE 3

Characteristics of fluorescence patterns of mouse embryos sexed under standard conditions.

H-Y-positive embryos	H-Y-negative embryos
Relatively large numbers of small discrete punctate spots randomly distributed over exterior surface	From dark to less clearly defined diffuse background
Areas of more diffuse fluorescence	Relatively small number of discrete punctate spots
Observed in both H-Y-positive and H-Y-negative embryos: Larger spots or blobs generally associated with dead or extruded materials In about one-third of all embryos fluorescent spots in perivitelline space	

TABLE 4

Changes in fluorescence patterns of mouse embryos after more intensive washing procedures.

H-Y-positive embryos	H-Y-negative embryos
Small discrete punctate spots tend to be more defined	Reduction of number of spots
Diffuse areas more localized	Reduction of background to none or to a very diffuse equal fluorescence
Observed in both H-Y-positive and H-Y-negative embryos: Larger spots or blobs more strictly associated with dead or extruded materials Spots in perivitelline space rare or no longer observed	

TABLE 5

Changes in fluorescence patterns of mouse embryos after performing assay at 4°C combined with longer washing steps.

H-Y-positive embryos	H-Y-negative embryos
Dark background	Very dark background
Increased number of finer spots with brighter intensity	Only a few, more fine spots
Spots more regularly distributed over the whole cell mass	

with 0.10 and 0.05% pronase for 5 and 10 min at 37°C and subsequent sexing resulted in more and more intensive staining compared with staining of control embryos without pronase treatment. The zona pellucida was highly fluorescent under these conditions. There were no non-fluorescent embryos. Although after pretreatment with 0.01% and even lower percentages of pronase fluorescence patterns resembled those on control embryos, intensive staining of the zona remained a major problem. As a consequence no discrimination could be made between H-Y-positive and H-Y-negative embryos after pronase treatment.

After fragmentation of the IgM monoclonal antibodies, fragments exhibited a reduced capacity to bind to H-Y antigen in a testis supernatant EIA (Booman et al., 1989) as compared with the intact antibodies. Not surprisingly, the mouse embryos sexed with a cocktail of fragmentated 5 XI, 4 VII and HY-1 showed only a very diffuse background fluorescence, caused by the secondary antibody. Results with a cocktail of the purified anti-H-Y monoclonal antibodies before fragmentation were about the same as with unpurified supernatant of antibody 5 XI alone. With the cocktail no improvement of positive signal was observed.

Modulation of the H-Y antigen

Performance of the indirect assay at 4°C resulted in many fluorescent spots in between the embryonic cells and the zona pellucida. By introducing prolonged washing steps this phenomenon could be prevented. The combination 4°C/longer washing steps led to a magnified positive fluorescence signal and a more clearcut discrimination between H-Y-positive and H-Y-negative embryos (Table 5). Addition of 0.1% NaN_3 to all media either in combination with 4°C or with 22°C gave about the same staining patterns as when performed at 4°C alone.

Amplification system

Staining with GAM-RPE as a secondary antibody was somewhat brighter than with GAM-FITC, and resulted in a more clearcut discrimination between positive and negative embryos. Fluorescence patterns were about the same as with GAM-FITC. Fluorescence of GAM-RPE, however, tended to bleach rather rapidly and both RPE preparations used bound somewhat non-specifically to the zona pellucida.

Pregnancy rate for both GAM-RPE treated embryos and control embryos

was about 20% (142 and 105 embryos were implanted, respectively) from which it can be concluded that RPE does not affect viability of embryos.

Evaluation of sexing bovine embryos under "improved" conditions

Most bovine embryos flushed at Day 6-7 were morulas and early blastocysts. The indirect fluorescence assay with improved conditions resulted in a somewhat less subjective assignment of sex because of a more marked difference between positive- and negative-staining embryos. However, as in mouse embryos, GAM-RPE bound non-specifically to the zona pellucida. This non-specific fluorescence might overshadow specific fluorescence of low intensity on the embryonic cells. As with sexing under standard conditions three categories of (non-)fluorescent embryos were distinguishable (Table 6).

A total of 96 bovine embryos were evaluated for expression of H-Y antigen; of these, 40 (42%) were classified fluorescent, 53 (55%) non-fluorescent and 3 (3%) non-sexable because of fluorescence of the zona. From 22 embryos a readable karyogram was obtained. Sixteen embryos (73%) were assigned correctly: 6 out of 6 (100%) as H-Y-positive classified embryos and 10 out of 16 (63%) as H-Y-negative classified embryos. Detailed results are given in Table 7, from which it can be concluded that nearly all misclassifications are in the category of H-Y-negative embryos. The larger number of non-fluorescent as compared to fluorescent embryos seems to be a consequence of the false H-Y negatives in that category. All fluorescent embryos were males. The intermediate group of embryos was relatively small compared with sexing under standard conditions.

TABLE 6

Characteristics of fluorescence patterns of bovine embryos sexed under improved conditions.

H-Y-positive embryos	H-Y-negative embryos	Intermediate category[a]
Patches of fluorescence randomly distributed over the exterior surface of the embryo Occasionally small lines of fluorescence around some cells Relatively high intensity of fluorescence	Negligible background No patches of fluorescence Occasionally very few small bright spots	Background of low intensity or Some fluorescent areas of low intensity or Several very fast bleached spots of low intensity

[a]Embryos with less clear staining characteristics; depending on intensity of fluorescence and size of fluorescent part of cell mass classified as fluorescent (H-Y positive) or non-fluorescent (H-Y negative).

TABLE 7

Numbers of embryos and accuracy of sex determination in each category of (non-)fluorescent bovine embryos sexed under improved conditions.

	No. of embryos observed		No. of readable karyotypes	No. of embryos sexed correctly		
	Fluorescent	Non-fluorescent		Fluorescent	Non-fluorescent	Total
H-Y-positive embryos	35(36%)	-	5	5/5(100%)	-	5/5 (100%)
H-Y-negative embryos	-	45(47%)	14	-	9/14(64%)	9/14(64%)
Intermediate embryos	5(5%)	8(8%)	3	1/1(100%)	1/2 (50%)	2/3 (67%)
Total	40(42%)	53(55%)	22(23%)	6/6(100%)	10/16(63%)	16/22(73%)

Discussion

Although bovine embryos could be sexed under standard conditions with an accuracy of 77%, evaluation of the staining patterns was fairly subjective. Sexing resulted in more false positives than false negatives. This is probably caused by non-specific binding of the monoclonal antibodies to the embryonic cells, which overshadows low intensity specific fluorescence. White et al. (1983) using monoclonal antibodies and Piedrahita and Anderson (1985) using polyclonal antibodies reported likewise that in murine embryos the proportion of embryos classified as H-Y positive tended to be larger than the proportion of embryos classified as H-Y negative. In addition, data reported by White et al. (1984) indicated a similar although non-significant trend with bovine embryos. If the monoclonal antibodies or most of the immunoglobulins in a polyclonal antiserum are directed to carbohydrate moieties, as might be the case according to Shapiro and Erickson (1981), slight cross-reactivity with another glycosylated protein present on the surface of both female and male cells could be an explanation for finding more fluorescent than non-fluorescent embryos. Also, Wachtel (1983) suggested that humoral H-Y antibodies may be major histocompatibility complex (MHC)-restricted and H-Y antibodies may therefore recognize H-Y antigen in association with MHC-determined antigens, which could result in some non-male-specific binding to female cells. Another cause of the false positives might be limited expression of H-Y antigen on female cells (Zenzes and Reed, 1984).

A more trivial cause for non-specific binding might be that the zona pellucida presents not only a barrier for the intrusion of anti-H-Y antibodies, but also for the diffusion of the unbound antibodies. The large molecular size of the IgM antibody might hamper its penetration through the zona, although Sellens and Jenkinson (1975) have reported that not only IgG, but also IgM molecules can penetrate the zona pellucida of mouse embryos. The rate of passage, however, may be critical. Prolonged washing steps indeed diminished non-specific binding. Acceleration of the diffusión process by making the zona more porous, however, did not succeed. Pronase has been reported to be effective in lysing zonae of mouse blastocysts (Bowman and McLaren, 1970). Although in our experiments mouse embryos were pretreated with much lower pronase concentrations than normally used for zona removal (0.5%), at all concentrations non-specific binding increased,

probably due to damage of the embryonic cells and the zona itself. Because a cocktail of fragments of monoclonal antibodies did not result in any specific fluorescence, it could not be clarified whether IgG-like fragments can more easily penetrate the zona than the intact IgM molecules. It may be that fragmentation resulted in damage of the antigen binding site and subsequent loss of binding capacity of the antibodies. Another possibility may be that modifications occur in that part of the Ig molecule that binds with secondary antibody.

With regard to the magnitude of the specific signal, modulation of the H-Y antigen seems to be partly responsible for the weak fluorescence when sexed under standard conditions. The phenomenon of antigenic modulation has been first described by Boyse et al. (1967). Modulation is the result of internalization of the antigen and the bound antibody. This is typically preceded by capping of the antibody-antigen complex, a process during which the complex appears to localize to one region of the cell surface. Capping is an active, temperature- and metabolically dependent process (Taylor et al., 1971). With mouse lymphocyte-surface immunoglobulin molecules capping was inhibited both at 0°C and at 20°C with NaN_3 or dinitrophenol. Modulation has also been described for H-2 antigens on the embryonic cell surface (Geib et al., 1976). Our results confirm those findings for the H-Y antigen. Because NaN_3 might harm viability of the embryos, working at 4°C will be preferable to inhibit the process of modulation. The low temperature might also prevent possible shedding of the H-Y antigen (Black, 1980). In spite of some disadvantages, as we have seen in our experiments, the use of other fluorescence markers like phycoerythrins can also contribute to magnification of the positive signal.

When applying results obtained with mouse embryos to bovine embryos, we have to keep in mind that in case of sexing under standard conditions, fluorescence patterns in mouse embryos differed from those in bovine embryos. The murine monoclonal antibodies might bind to the mouse embryos in a more non-specific manner because of cross-reactivity with related antigens or binding to possible Fc-receptors on the surface of embryonic murine cells. Furthermore, the under standard conditions regularly occurring spots of fluorescence within the perivitelline space of mouse embryos indicate a difference in permeability of the zonae pellucidae.

Nevertheless, sexing bovine embryos under improved conditions resulted in greatly reduced non-specific binding, a more clearcut discrimination

between H-Y-positive and H-Y-negative embryos, as well as in a smaller intermediate group of embryos. In addition, there was a shift from too many positives to only false negatives. This indicates that there is no expression of the H-Y antigen by female embryos, as Zenzes and Reed (1984) suggest, or at most at a very low level, and that no or very little cross-reactivity with MHC-determined antigens occurs, as has been suggested by Wachtel (1983). No false positives also means that the assay is male specific. Although the monoclonal antibody used in this experiment showed a very slight cross-reactivity with primary cultures of female fibroblasts and ovary cells (Booman et al., 1989), this appeared not to interfere with the fluorescence staining of embryos. The occurrence of false-negative embryos might be caused by a weak expression of the H-Y antigen and/or a low affinity of the monoclonal antibody for bovine H-Y antigen combined with a not fully amplified signal in the assay. In addition, the non-specific binding of the fluorescence marker to the zona might overshadow low intensity fluorescence on the embryonic cells.

With regard to the low affinity to the H-Y antigen, it has been proposed that the serological determinant of H-Y antigen consists of a carbohydrate chain with a galactose terminal residue (Shapiro and Erickson, 1981). Polysaccharides alone predominantly elicit IgM antibodies (see, e.g. Kannagi and Hakomori, 1986) with generally low affinity (Klein, 1982). It has also been observed that during the early development of the mouse, changes in cell-surface carbohydrates occur so that sialic acid masks molecules on the surface of trophoblasts at the time of implantation (Marticorena et al., 1983). Because of the likelihood of carbohydrate side chain modifications or variable biosynthetic reactions occurring during early cell-surface antigen expression in developing embryos, antibodies recognizing amino acid structural epitopes of H-Y antigen might be more appropriate as reagents for an immunological sexing test. Although the antibodies used in our experiments were selected on basis of their binding to both soluble and cell-surface H-Y antigen originating from different sources (Booman et al., 1989), for a highly discriminating sensitive assay selection on high affinity antibodies for protein structural determinants on bovine cell-surface H-Y antigen needs more attention.

In the study of White et al. (1983), in which they also used monoclonal antibodies against the H-Y antigen in a comparable indirect fluorescence assay, the overall accuracy in identifying the sex of mouse embryos was

81%. In mouse experiments at our laboratory a percentage of 72% was obtained (data not shown). Based on validated polyclonal antibodies the sex of bovine embryos was identified with an overall accuracy of 84% (White et al., 1984, 1987), compared with 77 and 73% obtained in our experiments using monoclonal antibodies. Apart from the limited availability of the polyclonal antiserum, the latter shows that polyclonal antibodies may prove useful. The better results obtained by White et al. might be a consequence of selection on quality of the embryos. In our experiments no selection on quality was made and quality is an important factor for interpreting results of the test. Selection on good quality embryos may also account for the high percentage of readable karyograms White et al. obtained (46-58% vs. 33-59% in the literature; see King, 1984). Since in embryo transfer programs a considerable part of the embryo population is of lower quality, an assay for sexing embryos has to include all these embryos. It remains to be seen whether the accuracy of the assay can be improved by preparation of high affinity monoclonal antibodies and by amplification of the positive fluorescence signal.

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CHAPTER 5

EFFECTS OF ANTI-PROGESTERONE MURINE MONOCLONAL ANTIBODIES ON PLASMA PROGESTERONE CONCENTRATIONS AND ON ANTI-MOUSE REACTIONS IN CYCLIC PIGS

P. Booman, J.M. Schakenraad*, R. Veerhuis, D.F.M. van de Wiel, W. Koops, M. Tieman and L. Kruijt

Research Institute for Animal Production "Schoonoord",
P.O. Box 501, 3700 AM Zeist (The Netherlands)

*Present address:
University of Groningen, Department of Histology and Cell Biology,
Oostersingel 69-1, 9713 EZ Groningen (The Netherlands)

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Abstract

The effect of murine anti-progesterone monoclonal antibodies on plasma progesterone levels was studied in cyclic pigs. Intravenous injection of increasing amounts of anti-progesterone antibodies resulted in a concomitant rise in levels of antibody-bound progesterone. At the same time a significant rise in plasma concentrations of total progesterone was observed immediately after administration of higher doses of antibodies. The net effect of progesterone binding by the monoclonal antibody was significant for the group of animals injected with at least 50 mg of antibody. The effect, however, was relatively small and more or less independent of the quantities of antibody administered. Plasma levels of circulating antibody capable of binding progesterone were still detectable at the end of the experimental period. On the other hand it appeared that in all animals, except the one injected with the largest quantity (500 mg) of antibody, the concentration of antibody-bound progesterone started to decrease as early as the first day after injection and was below detectable levels within one or two days. Mechanisms that may control the initial increase in progesterone levels and enable the animals to maintain adequate levels of free progesterone in circulation are discussed. Administration of a minimum dose of 32 mg antibody resulted in an anti-mouse immune response after the first injection, and subsequently in a neutralization of the anti-progesterone monoclonal antibodies immediately after the second injection. When smaller quantities of antibody ($\leq$ 20 mg) were used, anti-mouse antibodies were detected only after the second or third injection.

Introduction

The neutralization of hormones by specific antibodies has been extensively used to study endocrine systems in vivo. Active immunization against steroid hormones, for instance, may either enhance or limit fertility, particularly in females (Webb et al., 1984). Immunoneutralization by active immunization, however, is not always reproducible. Within species, and even within breeds, marked variations in the magnitude and speed of antibody response may occur. These variations may be circumvented by passive

immunization with monoclonal antibodies which, by definition, have identical characteristics.

Several passive immunization experiments with murine anti-progesterone monoclonal antibodies have been performed in mice and ferrets to study the ways by which progesterone acts on the female reproductive tract during the earliest stages of gestation (Wright et al., 1982; Wang et al., 1984; Rider et al., 1985; Rider and Heap, 1986). In mice and ferrets anti-progesterone antibodies can block implantation (Wright et al., 1982; Wang et al., 1984; Rider and Heap, 1986). The mechanism behind this phenomenon is still unclear. In general, it is believed that immunization results in a reduction of the amount of progesterone freely available in circulation to interact with target cell receptors. The reduction in fertility caused by the anti-progesterone antibodies can be reversed by administration of an equivalent amount of free hormone (Rider et al., 1985). However, in all experiments the concentration of total progesterone in plasma was manifold higher in the experimental than in the control group of animals. Although a reduction in free/total progesterone ratio was observed after administration of anti-progesterone antibodies, the absolute amount of free progesterone (i.e., not bound to binding proteins or antibody) increased at the same time (Cheesman and Chatterton, 1982; Nowak et al., 1986). A mechanism might be proposed by which transient decreases in levels of free progesterone, of which normally 95% is bound to proteins like albumin and glucocorticoid binding globulin (CBG) (Hoffmann et al., 1969), are compensated by a rapid dissociation of free progesterone from the CBG-progesterone pool.

In order to further elucidate the mechanism by which steroid levels are regulated, we studied the effect of murine anti-progesterone monoclonal antibodies on plasma levels of total and non-antibody-bound progesterone in cyclic pigs. Since murine monoclonal antibodies, when used in the pig, may elicit an anti-mouse immune response which might block their capacity to neutralize progesterone, we also investigated whether an anti-mouse immune response occurs after repeated administration. Part of this study has been published previously in abstract-form (Booman et al., 1984b).

Materials and Methods

Animals

Thirteen nulliparous female pigs (Dutch Landrace x Great Yorkshire), 7-9 months old and weighing ± 100 kg, were housed individually and fed according to their requirements. Occurrence of oestrus was checked twice daily by observation of vulval swelling and by exerting pressure on the back of the gilts. The first day of standing heat was defined as Day 0 of the oestrous cycle. After two regular cycles and at least 10 days before the experiment started, the animals were chronically cannulated as described by Van de Wiel and Eikelenboom (1977).

Monoclonal antibody

Monoclonal antibodies (MCAs) against progesterone were produced as described by Booman et al. (1984a). The immunogens used were progesterone-11α-hydroxy-hemisuccinate conjugated to bovine serum albumin (P-11α-BSA) and progesterone-7α-carboxyethylthioether conjugated to bovine thyroid globulin (P-7α-BTG). For our experiments antibody 4 XVIII, raised against P-11α-BSA was selected. This antibody showed low cross-reactivity with a large series of related steroids. In a double antibody heterologous solid phase enzyme immunoassay (EIA) the association constant (K_A) of the MCA for progesterone was 2×10^{10} l/mol (Booman et al., 1984a); it should be noted, however, that this K_A may be dependent on the type of assay (Van Weemen, 1974). Ascites fluid of cell line 4 XVIII was pooled and its titer (defined as the working dilution when tracer binds 50% of maximum binding in the EIA-system) determined at 1:13,500,000. Serum-free cell culture supernatant (HB 102; HANA Media) was produced and tenfold concentrated on an XM-50 Diaflo membrane (Amicon Corp.). The titer of the concentrated serum-free supernatant was 1:120,000.

The immunoglobulin M (IgM) concentration of both antibody preparations was determined by EIA. Briefly, microtiter plates were coated with rabbit anti-mouse Ig antiserum (RAM-Ig; Nordic Immunological Laboratories). Fourfold serial dilutions of purified mouse myeloma IgM (Sigma Chemicals) from 25 μg to 1.5 ng per 100 μl and of both antibody preparations were added and bound IgM was quantified by subsequent incubation with RAM-Ig conjuga-

ted to horse-radish peroxidase (HRP) (Nordic Immunological Laboratories). Detailed procedures for the EIA were similar to those described earlier (Booman et al., 1984a). Concentrations of IgM in ascites and serum-free supernatant were 100 and 2 mg/ml, respectively. Although the ascites fluid was produced in male mice, it appeared that clarified ascites contained about 3.7 μg progesterone per ml as determined by radio immunoassay (Van de Wiel et al., 1981); this progesterone is probably produced by the andrenal gland. In the concentrated serum-free supernatant no progesterone could be detected ($\leq$ 0.1 ng/ml).

Experimental design and blood sampling

Three different experiments were performed. In the first one, 6 gilts were injected intravenously on Day 12 $\pm$ 1 of the oestrous cycle with 10 ml aliquots of ascites fluid diluted with 0.9% NaCl, containing 50, 100, 200, 300, 400 and 500 mg IgM, respectively. Blood samples were collected once a day during the whole cycle and frequently on the day of MCA-administration according to the following scheme: at 155, 95, 65, 35 and 5 min before and at 2, 4, 6, 8, 10, 15, 20, 25, 35, 45, 55, 85, 115, 145, 175 and 205 min after administration and further on every hour for the next four hours. In the second experiment, 2 gilts were injected intravenously three times with 10 ml of concentrated serum-free supernatant containing 20 mg IgM with intervals of 14 days and starting on an arbitrary day of the cycle. Blood samples were taken once a day starting from at least 14 days before the first injection until 14 days after the last injection. The third experiment was similar to the first one, except that 4 gilts were injected with 10 ml aliquots of concentrated serum-free supernatant diluted with 0.9% NaCl, containing 2, 4, 8 and 16 mg IgM, respectively, and 1 gilt was injected with 16 ml of supernatant containing 32 mg IgM. In this experiment the injections of IgM were repeated during the next oestrous cycle, using the same IgM concentrations.

Blood samples were collected in heparinized tubes on ice and the plasma, separated by centrifugation at 1600g for 10 min at 4°C, was stored at -20°C.

Enzyme immunoassays

Total progesterone

After extraction of progesterone from plasma, total progesterone (P) was determined by EIA as described by Van de Wiel and Koops (1986). Plasma samples were diluted 1:10 with assay buffer and 20 μl of diluted sample was extracted with 1.75 ml of diethylether. The contents of each tube was mixed on a Vortex mixer and allowed to stand for at least 15 min at -30°C. The ether fraction was decanted into a clean tube and evaporated under a N_2-stream in a waterbath (40°C), after which 500 μl of assay buffer was added to each tube. The tubes were shaken gently (Vortex) and incubated for at least 30 min. Next, 100 μl aliquots of the extracts were incubated in duplicate in microtiter plate wells (Costar) coated with rabbit anti-P-7α-BSA antiserum. Standard amounts of progesterone (0-200 pg/ml, 100 μl/well) were incubated in quadruplicate. HRP conjugated to progesterone-6β-hydroxy-hemisuccinate (P-HRP) was used as a tracer.

Non-MCA-bound progesterone

In order to determine the plasma levels of non-MCA-bound progesterone, samples were precleared of monoclonal antibodies using ammonium sulphate precipitation (experiment 1) or absorption with donkey anti-mouse Ig coupled to cellulose (SacCel; Wellcome Research Laboratories) (experiments 2 and 3). The levels of progesterone were measured as described above. Because of the limited binding capacity of SacCel, plasma samples were diluted before absorption. Appropriate dilutions were determined beforehand by screening serial dilutions of plasma samples from every animal in an EIA for residual mouse IgM after SacCel absorption.

In order to correct for the dilution and a-specific loss of progesterone during the absorption procedures, the levels of non-MCA-bound P for each animal were corrected in such a way, that the mean levels of total P and non-MCA-bound P before the first injection were equal.

Circulating MCA capable of binding progesterone

Plasma levels of circulating MCA still capable of binding progesterone (MCA - (P~MCA)) were determined in the EIA as described by Booman et al. (1984a). Briefly, microtiter plates were coated with RAM-Ig. After incubation with the plasma samples, bound MCA capable of binding progesterone

was detected by incubation with P-HRP.

Anti-mouse MCA response

Microtiter plates were coated with anti-progesterone monoclonal antibody 4 XVIII (1.8 μg IgM/ml, 100 μg/well). Plasma samples were added and the assay developed with rabbit anti-swine Ig conjugated to HRP (Nordic Immunological Laboratories). Detailed assay procedures for the EIA were similar to those described earlier (Booman et al., 1984a).

Statistical analysis

Means and corresponding standard deviations were calculated. Levels of total progesterone and non-MCA-bound progesterone before and at different time periods after injection were compared with the sign-test. Unless stated otherwise, a two-sided test with a level of significance of 0.05 was used.

Results

Plasma levels (means $\pm$ s.d.) of total progesterone, non-MCA-bound progesterone and MCA-bound progesterone (P~MCA) from the gilts in the first experiment immediately before and at different time periods after injection of various quantities of IgM from ascites fluid are listed in Table 1. P~MCA was calculated as the difference between total P and non-MCA-bound P. The results indicate that anti-progesterone MCAs do bind to progesterone in vivo. In general, increasing quantities of IgM administered correlate with increasing amounts of progesterone bound to MCA. Plasma levels of total P and non-MCA-bound P could again be superimposed after a period of 24 h, with exception of the gilt injected with the largest quantity of IgM (500 mg) in which non-MCA-bound P levels equaled those of total P after about 7 days (Fig. 2).

Immediately after injection of the MCAs there is a significant rise ($p < 0.05$) in plasma levels of total P, with a higher rise if more antibody is injected. P~MCA, calculated as the difference between total P and non-MCA-bound P, gives a good estimate of the actual amount of progesterone bound by MCA. However, in order to determine the net effect of progesterone

TABLE 1

Plasma levels (means ± standard deviations) of total progesterone (T P), non-MCA-bound P (NB P) and MCA-bound P (P-MCA) in ng/ml before and at different periods after injection of anti-progesterone monoclonal antibodies.

No. of gilt	MA 12			MA 9			F 18			F 15		
quantity of IgM (mg) (ascites fluid)	50			100			200			300		
	T P	NB P	P-MCA	T P	NB P	P-MCA	T P	NB P	P-MCA	T P	NB P	P-MCA
before injection	37.3±5.6	38.8±1.8		26.7±2.5	26.0±2.3		31.8±1.7	33.6±2.8		15.9±1.3	15.8±2.4	
period after injection (h)												
0 - 0.5	40.2±6.4	33.9±5.9	6.3±3.2	34.2±2.9	25.1±4.5	9.1±4.7	41.7±5.4	22.6±3.8	19.1±4.7	32.7±2.1	13.8±2.1	18.9±2.2
0.5 - 3	30.3±2.9	26.4±3.7	3.9±3.8	29.7±2.9	22.9±2.6	6.8±3.2	32.8±1.5	20.5±3.0	12.3±3.7	32.5±6.3	11.6±1.3	20.9±5.5
3 - 8	35.7±1.4	33.0±1.7	2.7±3.0	32.8±2.2	27.5±3.0	5.3±3.5	32.3±3.3	24.8±3.8	7.5±3.0	16.5±2.6	5.5±1.2	11.0±1.8
0 - 8	34.3±4.2	30.4±3.5	4.0±2.0	32.1±2.4	25.5±2.3	6.7±2.3	33.5±4.0	22.8±2.7	10.7±5.2	22.8±8.9	8.4±3.6	14.4±5.2

No. of gilt	MA 10			MA 2		
quantity of IgM (mg) (ascites fluid)	400			500		
	T P	NB P	P-MCA	T P	NB P	P-MCA
before injection	37.3±2.3	38.4±2.0		38.0±2.0	39.9±1.9	
period after injection (h)						
0 - 0.5	51.0±2.3	37.0±6.0	14.1±5.3	59.4±5.1	34.1±3.5	25.3±3.1
0.5 - 3	45.4±4.9	35.0±3.0	10.3±5.3	53.2±4.0	31.1±4.9	22.0±2.6
3 - 8	43.5±5.1	40.1±5.1	3.4±1.8	54.4±2.2	26.4±1.4	28.0±2.6
0 - 8	45.0±5.3	38.3±4.7	6.7±5.0	55.1±3.1	29.0±4.0	26.1±3.4

Significant differences ($p < 0.05$, two-sided, sign test) of T P and NB P in periods after injection with respective levels before injection:

	T P	NB P
0 - 0.5	*	*
0.5 - 3		*
3 - 8		
0 - 8		*

TABLE 2

Plasma levels (means ± standard deviations) of total progesterone (T P), non-MCA-bound P (NB P) and MCA-bound P (P-MCA) in ng/ml before and at different periods after injection of anti-progesterone monoclonal antibodies.

No. of gilt	IF 5			IF 12			IF 13			IF 3			IF 9		
quantity of IgM (mg) (conc. serum-free supernatant)	2			4			8			16			32		
	T P	NB P	P-MCA	T P	NB P	P-MCA	T P	NB P	P-MCA	T P	NB P	P-MCA	T P	NB P	P-MCA
before first injection	40.8±2.7	42.3±3.9		23.3±2.6	23.6±2.9		22.5±2.0	23.9±1.2		40.2±3.0	43.4±0.6		19.0±3.1	18.7±2.6	
period after first injection (h)															
0 - 0.5	42.7±4.6	40.0±3.7	2.7±6.4	19.5±2.0	19.9±2.2	-0.4±2.4	24.2±2.6	26.0±4.3	-1.8±3.6	40.4±8.8	37.8±5.3	2.6±10.7	21.0±3.2	18.2±2.8	2.7±2.2
0.5 - 3	39.6±3.4	40.9±3.8	-1.3±5.7	16.9±2.3	17.8±1.3	-0.9±2.0	31.5±2.1	22.6±3.4	8.9±4.9	36.7±2.6	35.2±4.5	1.6± 5.4	24.2±1.8	19.9±2.9	4.4±1.5
3 - 8	35.8±5.0	36.1±7.6	-0.3±5.9	15.1±1.4	15.7±1.7	-0.6±2.9	26.2±5.7	26.4±3.1	-0.1±4.2	46.2±6.7	42.0±2.3	4.2± 6.3	17.3±4.7	17.6±6.5	-0.3±2.3
0 - 8	37.7±4.8	38.0±6.1	-0.3±4.5	16.1±2.1	16.9±2.0	-0.8±2.2	27.0±4.9	25.0±2.3	2.0±5.6	42.3±6.3	39.2±4.2	3.0± 5.1	19.6±5.0	18.0±4.8	1.6±2.2
before second injection	31.5±7.3	27.9±2.9		17.8±2.3	17.2±2.6		23.0±3.5	23.1±1.1		41.0±3.4	40.4±3.4		22.5±1.3	22.8±0.3	
period after second injection (h)															
0 - 0.5	28.7±2.5	32.6±3.0	-3.9±3.0	18.9±3.6	15.8±3.1	3.1±2.3	23.7±4.3	21.2±2.5	2.5±6.3	42.6±3.6	39.9±4.8	2.7± 4.8	25.1±2.5	24.6±2.8	0.5±1.8
0.5 - 3	31.8±2.5	27.1±2.4	4.8±2.8	19.8±2.9	17.9±2.4	1.9±3.6	28.0±6.8	20.3±1.3	7.7±6.5	37.2±5.3	33.5±4.5	3.7± 6.1	19.7±6.6	20.0±6.5	-0.3±0.7
3 - 8	31.2±1.9	33.1±5.1	-1.9±4.8	17.1±1.5	17.1±1.5	0.0±2.6	16.0±3.1	17.1±1.8	-1.0±4.4	34.7±5.6	37.4±8.0	-2.7± 3.8	17.8±3.3	18.5±2.9	-0.6±2.7
0 - 8	30.9±1.4	31.0±4.6	-0.1±5.2	18.0±1.8	17.2±1.9	0.7±2.8	20.3±6.4	18.8±1.9	1.5±5.2	37.0±4.5	37.0±6.3	0.0± 5.1	19.3±3.7	19.8±3.4	-0.5±2.1

binding by MCA, the difference between non-MCA-bound P immediately before and at different periods after injection was calculated. The levels of non-MCA-bound P at all time periods after injection are significantly reduced ($p < 0.05$) compared to values obtained before injection, with exception of the 3-8 h period. The results presented in Table 1 suggest that this reduction, or the net effect of progesterone binding by MCA, is more or less independent of the quantities of IgM injected.

A rise in plasma levels of circulating MCA capable of binding progesterone was observed immediately after injection. The MCAs were still detectable by EIA at the end of the experimental period. As typical examples of this experiment, progesterone and MCA levels of two gilts are shown in Figs. 1 and 2.

In the second experiment the first blood sample was taken 24 hours after injection, when according to the results of experiment 1 the transient increase in total P as well as the difference between total P and non-MCA-bound P had subsided. Both gilts generated an anti-mouse immune response after injection with the MCAs. In gilt Ac 1 a rapid increase in the amount of antibodies against the mouse MCA can be detected after the second injection. This increase continues after the third injection (Fig. 3). In the other gilt anti-mouse levels do not rise until 2 days after the third injection (= 16 days after the second injection). In Fig. 3 plasma levels of total P, non-MCA-bound P, circulating MCA capable of binding P and the anti-mouse immune response are shown for one of the two gilts (Ac 1) in this experiment.

Data from the third experiment are listed in Table 2. In the gilts injected with concentrated serum-free supernatant no significant increase in levels of total P occurs immediately after injection ($p = 0.054$, one-sided). As in experiment 1, the net effect of progesterone binding by MCA is calculated as the difference between levels of non-MCA-bound P before and after injection. No significant ($p < 0.05$) difference could be found in this respect.

Results of experiments 1 and 3 have been combined in Table 3, showing the amounts of progesterone that theoretically can be bound by the antibodies as compared to the amounts of actually bound progesterone (P~MCA) in the period 0-0.5 h after injection.

In the third experiment a minimum injected dose of 8 mg IgM results in a detectable rise in MCA capable of binding progesterone. As early as

TABLE 3

Amounts of progesterone (P) theoretically and actually bound by the anti-P monoclonal antibody[a].

mg IgM anti-P administered	µg IgM anti-P/ml plasma[b]	total P before administration[c] (ng P/ml plasma)	amount of P that can maximally be bound by MCA (theoretically[d]) (ng P/ml plasma)	% of P that can maximally be bound by MCA (theoretically[e])	amount of P actually[f] bound by MCA (ng P/ml plasma)	% of P actually[g] bound by MCA
2	0.4	40.8±2.7	1.3	3.2	2.7± 6.4	6.6±15.7
4	0.8	23.3±2.6	2.6	11.2	-0.4± 2.4	-1.7±10.4
8	1.6	22.5±2.0	5.2	23.1	-1.8± 3.6	-8.0±16.1
16	3.2	40.2±3.0	10.5	26.1	2.6±10.7	6.5±26.6
32	6.4	19.0±3.1	21.0	110.5	2.7± 2.2	14.2±11.6
50	10	37.3±5.6	32.8	87.9	6.3± 3.2	16.9± 8.7
100	20	26.7±2.5	65.6	245.7	9.1± 4.7	34.1±17.8
200	40	31.8±1.7	131.2	412.6	19.1± 4.7	60.1±14.7
300	60	15.9±1.3	196.8	1237.7	18.9± 2.2	118.9±13.8[h]
400	80	37.3±2.3	262.4	703.5	14.1± 5.3	37.8±14.7
500	100	38.0±2.0	328.0	863.2	25.3± 3.1	66.6± 8.1

[a]Based on results obtained from exp. 1 and 3, and presented in Tables 1 and 2 (first injection), respectively.
[b]Based on a total plasma volume of 5 liters.
[c]Means ± standard deviations of samples taken before administration of the MCA.
[d]Based on the data of column 2 and on the assumptions of 10 active binding sites/molecule IgM and a MW of 960,000 for IgM and 314.47 for progesterone.
[e]Based on the data of columns 3 and 4.
[f]Calculated as total P minus non-MCA-bound P at 0-0.5 h period after administration of the MCA (P-MCA in Tables 1 and 2).
[g]Based on the data of columns 3 and 6.
[h]The percentage of P actually bound by the anti-P MCA can surmount the 100 % because of a doubling in total P level at 0-0.5 h period after administration of the MCA, compared to total P before injection.

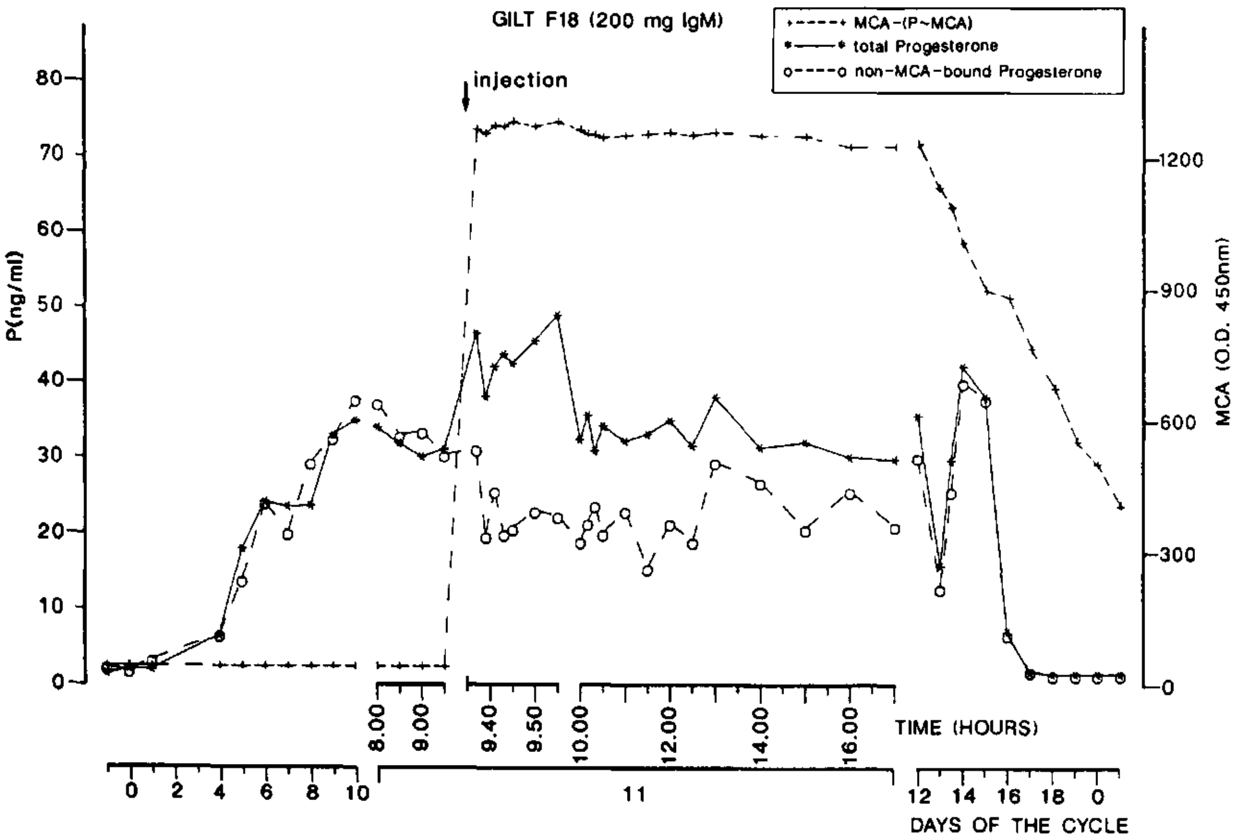

Fig. 1. Plasma levels of total progesterone, non-antibody-bound progesterone and antibody capable of binding progesterone before and after injection of anti-progesterone monoclonal antibodies.

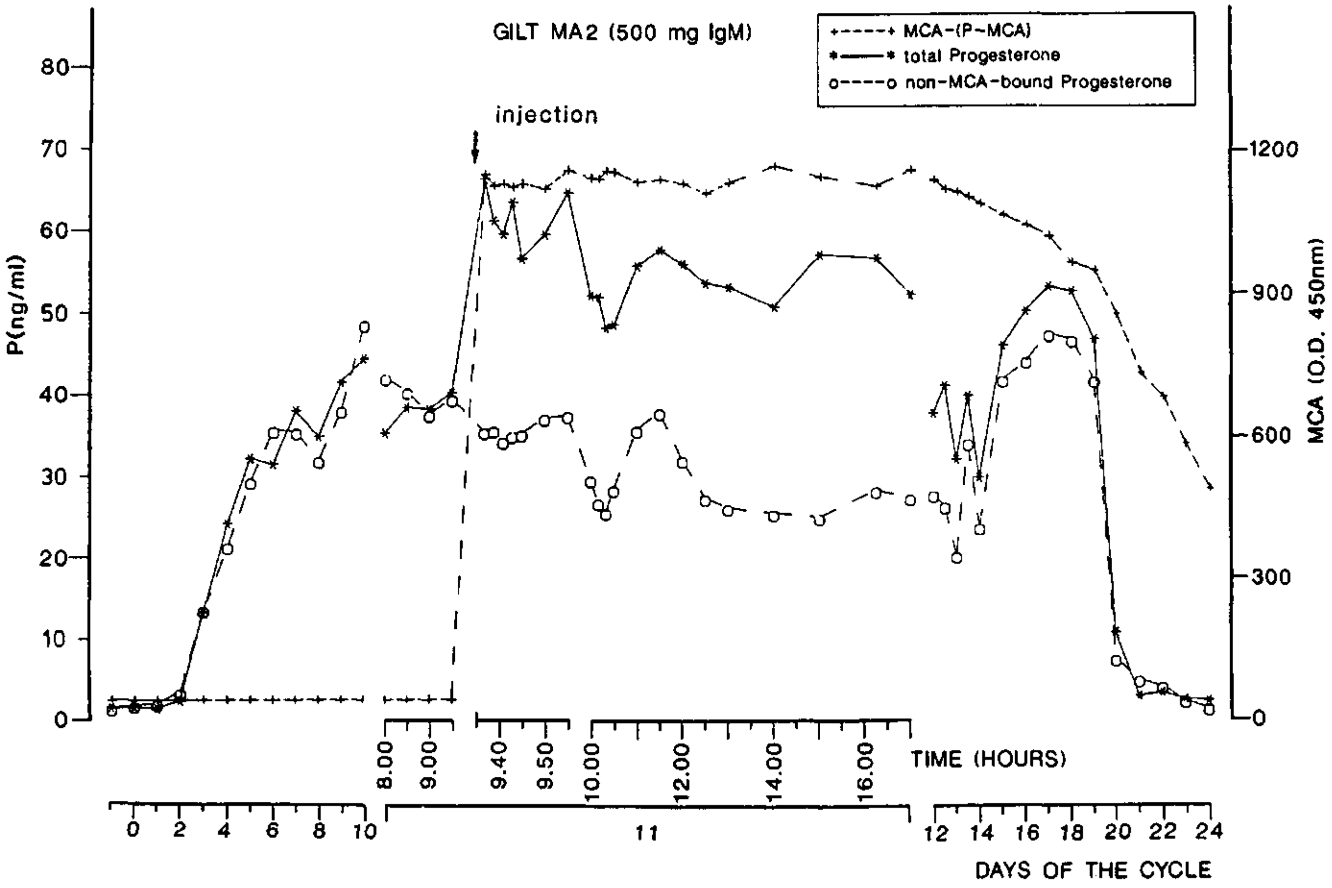

Fig. 2. Plasma levels of total progesterone, non-antibody-bound progesterone and antibody capable of binding progesterone before and after injection of anti-progesterone monoclonal antibodies.

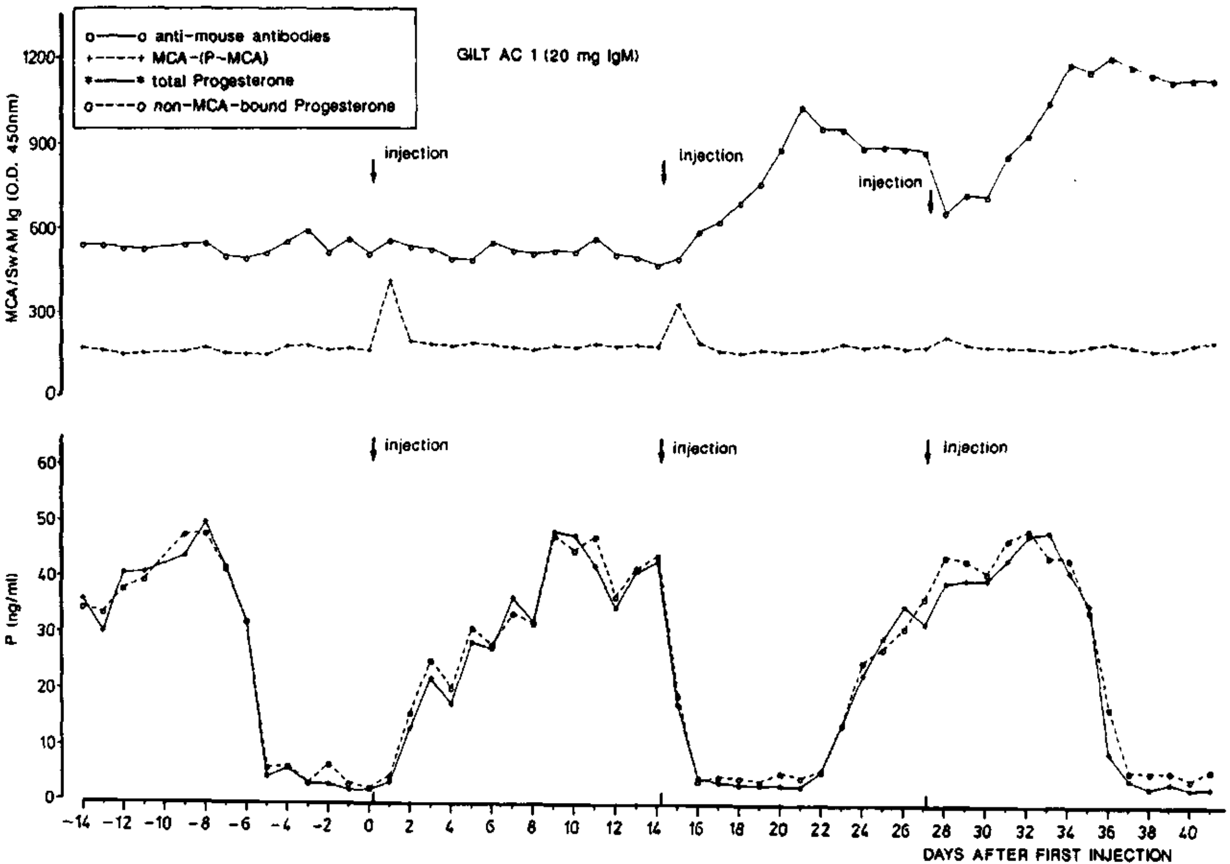

Fig. 3. Plasma levels of total progesterone, non-antibody-bound progesterone, antibody capable of binding progesterone and anti-mouse antibodies before and after repeated injection of anti-progesterone monoclonal antibodies.

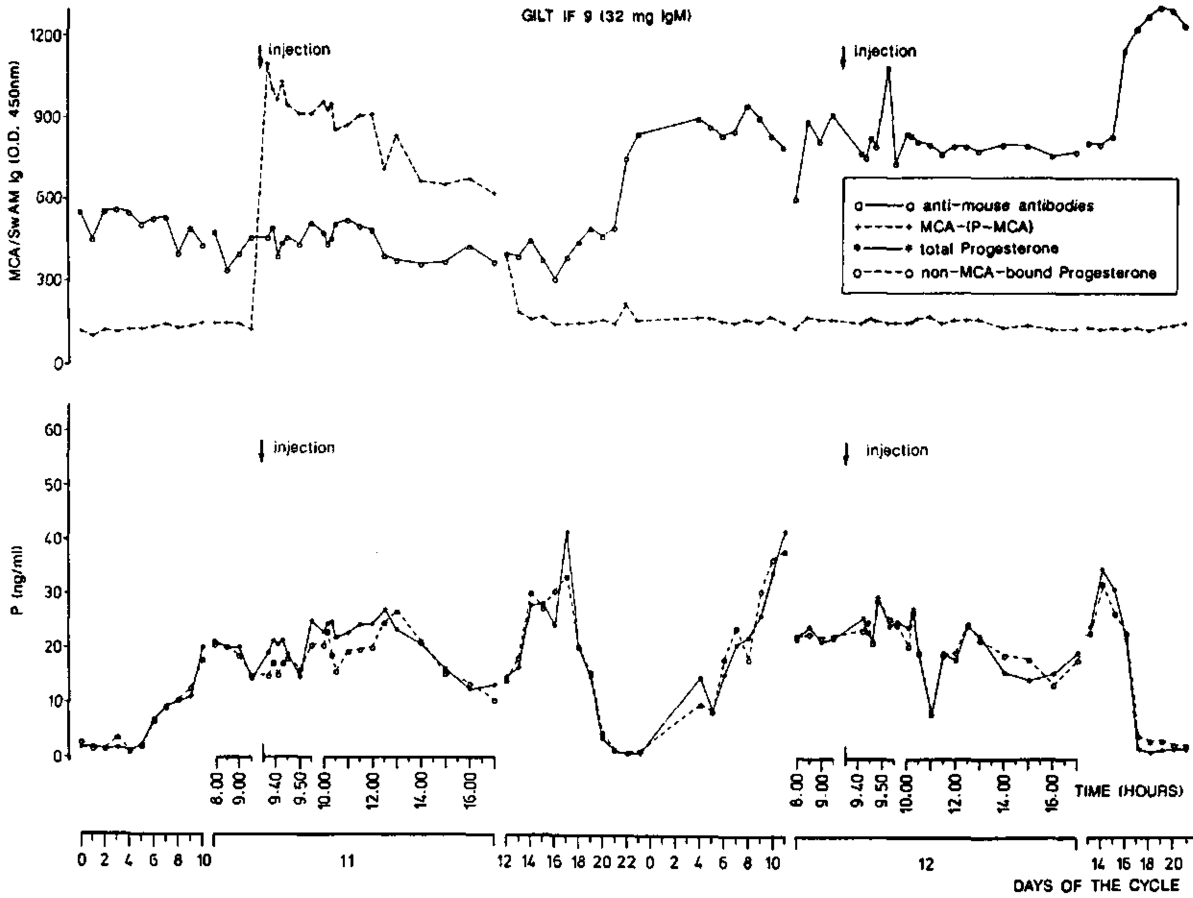

Fig. 4. Plasma levels of total progesterone, non-antibody-bound progesterone, antibody capable of binding progesterone and anti-mouse antibodies before and after repeated injection of anti-progesterone monoclonal antibodies.

the first day after administration 'unbound' MCA levels begin to decline, reaching preinjection levels after two days. In the animal injected with 32 mg of IgM no rise in unbound MCA can be observed after the second injection (Fig. 4). In this gilt anti-mouse antibodies can be detected in plasma 10 days after the first injection. After the second injection the anti-MCA response becomes very pronounced. A very slight anti-mouse response is seen after the first injection in the gilt that was treated with 8 mg IgM, which response becomes more evident after the second injection. No clear responses of anti-mouse Ig after either injection could be observed in the gilts injected with 2, 4 or 16 mg of IgM.

Discussion

Immunization with anti-steroid MCAs may modulate the reproductive function in domestic animals. To further elucidate the mechanism by which steroid levels are regulated, in the present study the efficacy of anti-progesterone monoclonal antibodies on the possible reduction of plasma progesterone levels in cyclic pigs was examined. Results from the first experiment, in which large amounts of IgM monoclonal antibodies directed against progesterone were administered, indicate that the antibodies do bind to progesterone in vivo. Administration of increasing amounts of antibody resulted in dose-correlated rises in amounts of progesterone actually bound by the antibody. However, although theoretically enough antibodies had been administered to bind most or all progesterone in circulation (88 to 1238%, Table 3), non-MCA-bound P was not reduced to basal levels.

In all animals, except the one injected with 500 mg IgM, the concentration of antibody-bound progesterone started to decrease on the first day of injection. No antibody-bound progesterone, calculated as the difference between total and non-MCA-bound P, could still be observed within one or two days after administration. The antibody-bound progesterone immune complexes, together with antibodies still capable of binding progesterone, were relatively rapidly cleared from the circulation. In addition, an equilibrium was established between the free steroid, steroid bound to antibody, and steroid bound to binding proteins. The discrepancy between the presence of circulating anti-progesterone antibodies and the absence of antibody-bound progesterone suggests that a substantial number

rect resorption of progesterone from body tissues with a subsequent increase in total progesterone levels in the circulation. Such a release of progesterone from body tissues would be an acceptable explanation for the observed increase in progesterone levels that parallels the increasing quantities of administered antibodies in our experiments, especially since it is known that in the pig about 200 times more progesterone is stored in the adipose tissue than the amount present in total blood, corresponding to the daily production of the corpora lutea of a sow on Day 11 of the cycle (Hillbrand and Elsaesser, 1983).

Nowak et al. (1986) and Cheesman and Chatterton (1982) found that antibody administration decreases the percentage, but increases or maintains the absolute amount of free progesterone present in circulation (i.e. not bound to binding proteins or antibody). These data seem to point to a mechanism which enables the animal to maintain adequate levels of free progesterone in circulation. The rapid decline in levels of antibody-bound progesterone observed in our present experiments supports the concept of such a mechanism.

On the other hand, this suggested mechanism of the animal to maintain its progesterone levels seems to contradict the observations of a.o. Wang et al. (1984), Rider and Heap (1986) and Phillips et al. (1988) that anti-progesterone monoclonal antibodies can block pregnancy in mice, ferrets and rats. Recent studies (Wang et al., 1988), however, have revealed that only at a critical stage in early development the administration of large amounts of anti-progesterone antibody may locally inhibit the uterine uptake of progesterone, which in turn prevents implantation in mice.

The second and third experiment show that repeated administration of relatively small amounts of monoclonal antibodies may elicit an anti-mouse response in the pig. In the gilt injected with 32 mg of MCA, a clear anti-mouse response was already observed after the first injection. The anti-MCA antibodies may be an explanation for the observation that no MCA capable of binding progesterone was detectable after the second injection with 32 mg of antibody. Phillips et al. (1988) did not observe a decreased efficacy of anti-progesterone monoclonal antibodies after repeated administration in rats, although much more antibody had been injected (on a dose per kg body weight basis) than in our experiments. This discrepancy may be due to the greater homology between immunoglobulins of mouse and rat than of mouse and pig, resulting in a more pronounced anti-mouse Ig res-

of IgM binding sites remains unoccupied, as was previously observed by Ellis et al. (1988). They found that only 1-4% of the IgM sites is occupied by progesterone.

Administration of anti-progesterone monoclonal antibodies resulted in a significant rise in plasma concentrations of total P. Progesterone from the ascites fluid itself may have contributed to the increase in total P concentration after injection for only a small part. Based on 3.7 μg P/ml ascites fluid and on a total plasma volume of 5 liters, 1 ml ascites may maximally account for a rise in total P of 0.7 ng/ml plasma. Although the net effect of binding progesterone by antibody was significant, the effect was relatively small (0.1 - 10.9 ng/ml) and, within the range of 50-500 mg IgM, more or less independent of the quantities of IgM injected.

In the third experiment no significant rise in levels of total P nor a clear reduction of non-MCA-bound P could be observed. Theoretically, 110.5% of progesterone present in circulation can maximally be bound in the animal that was injected with 32 mg IgM (Table 3). Nevertheless, the quantities of antibody administered in the third experiment seem to be too small to result in detectable levels of antibody-bound progesterone, indicating that an immediate equilibrium is established between a low percentage of occupied binding sites and a relatively rapid clearance of antibody-bound progesterone.

Increased plasma concentrations of total progesterone, similar to the ones observed in the gilts injected with 50 mg antibody or more (experiment 1), were also found by Cheesman and Chatterton (1982) after treatment of rats with polyclonal antiserum against progesterone and, amongst others, by Wang et al. (1984), Rider and Heap (1986) and Phillips et al. (1988) after postcoital administration of anti-progesterone monoclonal antibodies in mice, ferrets and rats, respectively. Wang et al. (1984) suggested that a high concentration of total progesterone is maintained in circulation on account of the high affinity of the antibody for the steroid and that ovarian progesterone secretion had been stimulated by a pituitary or placental luteotrophin. Cheesman and Chatterton (1982), however, concluded from their experiments that progesterone levels were primarily controlled by binding of progesterone to the antibody rather than by stimulation of ovarian progesterone secretion, since the total progesterone concentration never exceeded the binding capacity of the circulating antibody. They suggested that administration of anti-progesterone antibodies leads to a di-

ponse in the pig compared to the rat. The anti-mouse response found in our experiments is in line with human clinical trials with murine monoclonal antibodies, in which almost all non-immunocompromised patients developed anti-mouse immunoglobulin antibodies which inhibited the therapeutic effect (for references, see Cole et al., 1985). A possible strategy to avoid such an anti-mouse response is the use of homologous monoclonal antibodies. However, these may, in turn, elicit an anti-idiotype response that, likewise, could reduce the effects of the MCA, although Ehrlich et al. (1987) reported that the repeated use of human MCA in rhesus monkeys did not trigger any other response than a normal decay in antibody level in all but one animal. At our laboratory a comparative study on the use of homologous and heterologous monoclonal antibodies for passive immunization is in progress.

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CHAPTER 6

CONSTRUCTION OF A BOVINE-MURINE HETEROMYELOMA CELL LINE; PRODUCTION OF BOVINE MONOCLONAL ANTIBODIES AGAINST ROTAVIRUS AND PREGNANT MARE SERUM GONADOTROPHIN

P. Booman[a], M. Tieman[a], D. van Zaane[b], A.A. Bosma[c] and G.F. de Boer[b]

[a]Research Institute for Animal Production "Schoonoord",
P.O. Box 501, 3700 AM Zeist (The Netherlands)
[b]Central Veterinary Institute, Virology Department,
P.O. Box 365, 8200 AJ Lelystad (The Netherlands)
[c]Department of Functional Morphology, Faculty of Veterinary Sciences,
State University, P.O. Box 80.157, 3508 TD Utrecht (The Netherlands)

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Abstract

Bovine-murine heteromyeloma cell lines were prepared by fusing lymphoid cells from a bovine leukemia virus-infected cow with mouse myeloma cells. Selection of hybrid cell colonies was based on the ratio of bovine and murine chromosomes, the presence of cell-surface immunoglobulins and growth characteristics. First generation fusion partners were compared for fusion efficiency and the generated number of antigen-specific antibody-producing clones. From first generation heteromyelomas hybrid cell colonies, which initially secreted antibodies, were selected to function as second generation fusion partners. Although fusion efficiencies for both generations did not differ, the second generation heteromyelomas yielded more specific antibody-producing clones. Fusion of heteromyelomas with either lymph node cells or splenocytes indicated that fusion with lymph node cells results in more specific antibody-producing clones, whereas fusion efficiency was found to be higher with splenocytes. The optimal time-intervals between the final booster injection and fusion were found to be 4 days for splenocytes and 7 days for lymph node cells, respectively. Finally, the characterization of bovine monoclonal antibodies against bovine rotavirus and pregnant mare serum gonadotrophin as well as their neutralizing capacity in vitro are described.

Introduction

In animal production monoclonal antibodies have become important tools in diagnostic and fundamental research. In addition, monoclonal antibodies can be used to optimize physiological processes such as growth and reproduction and are important in the treatment of certain animal diseases (Booman, 1988). A limiting factor for the use of monoclonal antibodies in passive immunization might be their murine or rat origin when applied in other species. Human clinical trials with murine monoclonal antibodies indicate that almost all non-immunocompromised patients developed anti-mouse immunoglobulin antibodies which inhibited the therapeutic effect (Cole et al., 1985). In pigs, repeated administration of murine antibodies generated a significant antibody response to mouse immunoglobulins (Arriëns and Booman, 1989). In addition, immunoglobulins have species-specific ef-

fector determinants, which allow cooperative cellular effects not possible with heterologous antibodies (Nose and Wigzell, 1983).

The production of antibodies other than of murine or rat origin has been greatly hampered, however, by the lack of suitable myeloma fusion partners. Nevertheless, some success has already been achieved in the development of monoclonal antibodies from livestock species. Srikumaran et al. (1983/1984) demonstrated the potential of interspecies hybridomas, produced by fusing mouse myeloma cells with bovine lymphocytes. Such interspecies fusions resulted in bovine antibodies against bovine enteric coronavirus (Raybould et al., 1985) as well as in antigen-specific ovine monoclonal antibodies (Beh et al., 1986; Groves et al., 1987b). A severe limitation to interspecies hybridomas, however, is their genetic instability, due to the selective elimination of the non-murine chromosomes. In another approach, followed by Tucker et al. (1984; 1987), Groves et al. (1987a; 1988) and by us, bovine-murine hybrid myelomas (heteromyelomas) have been constructed in an attempt to obtain a better fusion partner for the production of bovine monoclonal antibodies. It was anticipated that a heteromyeloma would retain the superior fusion characteristics of the mouse myeloma cells and be better able to support stable bovine antibody production because of the presence of bovine chromosomes.

The present paper reports the construction and selection of a bovine-murine heteromyeloma cell line. In addition, the optimal interval between boosting and harvesting of the lymphocytes and the source of lymphocytes was studied to a limited extent. In this study bovine monoclonal antibodies have been produced against rotavirus and pregnant mare serum gonadotrophin (PMSG).

Materials and Methods

Construction and selection of 1st generation heteromyeloma cell lines

Bovine-murine heteromyeloma cell lines were constructed by fusing lymphoid cells from a bovine leukemia virus (BLV)-infected cow with cells of the X63-Ag8.653 mouse myeloma cell line (Kearney et al., 1979). Seven years before, this cow was experimentally infected with BLV strain J 5 (Ressang et al., 1974). The supposedly transformed lymphoid cells were

selected from hyalin-appearing spots of the popliteal lymph node. After fusion, growth characteristics like doubling time, viability after freezing and subsequent thawing, and homogeneity of cell type of the colonies of hybrid cells were determined. In addition, the ratio between numbers of bovine and murine chromosomes, bovine and mouse cell-surface Ig, and secretion of bovine Ig were assessed. Selected hybridoma colonies were cloned twice by limiting dilution and the subclones checked on chromosomal content, cell-surface Ig and growth characteristics. Subclones chosen to be fusion partners were made HAT-sensitive by growing them for at least 2 months in medium supplemented with 30 μg/ml 8-azaguanine (Sigma). Cells were tested at intervals for HAT-sensitivity by culturing them in HAT-medium. The fusion partners were screened for the presence of BLV by procedures as described below.

Next, heteromyeloma cell lines were each fused in two consecutive experiments with popliteal lymph node cells from calves immunized against rotavirus. Heteromyeloma cell lines were selected on basis of fusion efficiency, number and stability of specific anti-rotavirus bovine Ig-producing clones 6 weeks after fusion, as well as earlier mentioned parameters. Fusion efficiency was defined as the number of colonies per 1 x 10^7 lymphocytes 3 weeks after fusion. Colonies were counted using an inverted microscope.

Comparison of 1st and 2nd generation heteromyeloma cell lines

From foregoing fusion experiments of the 1st generation heteromyelomas with lymphoid cells of calves immunized against rotavirus hybrid cell colonies, which initially secreted antibodies, were selected to function as 2nd generation bovine-(bovine-murine) heteromyelomas. Cells of those colonies were cloned twice and rendered HAT-sensitive. In order to compare the thus constructed 2nd generation of heteromyelomas with the 1st generation, fusion experiments with cells of popliteal lymph nodes of calves immunized against rotavirus were performed.

Timing of fusion and source of lymphocytes

The ultimately selected heteromyelomas were used for fusion experiments with both splenocytes and popliteal lymph node cells on Day 7 or Day 4

after the last booster injection, respectively. For these experiments calves were immunized with PMSG.

Immunization of calves

Male Jersey calves aged between 6 and 12 months were injected intramuscularly (i.m.) with 300 μg density gradient-purified bovine rotavirus (Dutch field isolate 26B) in complete Freund's adjuvant (CFA), followed by an injection of the similar dose (i.m.) in incomplete Freund's adjuvant (IFA) 3 weeks later. About 6 weeks later and 4 days before fusion, calves received 300 μg rotavirus in 10 ml phosphate buffered saline (PBS) intravenously. The purification procedure for rotavirus was as follows: Rotavirus was grown on MA 104 cells. After one cycle of freezing and thawing cell debris was removed by centrifugation for 10 min at 1500 rpm. The supernatant was loaded on 10 ml of 45% w/v sucrose/PBS. After centrifugation for 3 h at 27,000 rpm, the pellet was suspended in 5 ml of PBS and centrifuged once more for 15 min at 1500 rpm to remove insoluble material. The supernatant was used for immunization.

For immunization to PMSG, calves were injected five times i.m. with 3000 IU PMSG (Intervet) and adjuvant with intervals of 4 weeks. CFA was used in the first injection, and IFA thereafter. Calves were boosted intravenously with 3000 IU PMSG in 10 ml PBS about 6 weeks after the last injection and 7 or 4 days before fusion.

Fusion

Heteromyeloma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin, streptomycin and glutamin. Fusions between spleen or lymph node cells and myeloma cells were performed as previously described (Booman et al., 1984) with minor modifications. When in a later stage assays for bovine Ig and determination of Ig isotype had to be performed, hybrid cells were grown in DMEM with 10% horse serum.

ELISAs

Detection of bovine Ig

A double antibody sandwich assay was used for the detection of bovine Ig (Van Zaane and IJzerman, 1984). Briefly, microtiter plates were coated with a monoclonal antibody recognizing a common determinant of bovine IgG_1, IgG_2, IgA and IgM. After incubation with the culture supernatants bound antibody was detected using a mixture of rabbit antisera against bovine IgG, IgM and IgA conjugated to horse-radish peroxidase (HRP).

Determination and quantification of bovine Ig isotypes

In a similar type of assay, plates coated with monoclonal bovine Ig isotype-specific antibodies were used (Van Zaane and IJzerman, 1984) for the determination and quantification of bovine Ig isotypes. Two-fold dilutions of bovine Ig isotype standards and culture supernatants were added and the assay developed as described before. The concentration of bovine Ig in culture supernatants was estimated from titration curves.

Detection of anti-rotavirus bovine antibodies

An antibody capture assay was used for detection of specific anti-rotavirus antibody-secreting clones (Van Zaane and IJzerman, 1984). Microtiter plates were coated with the anti-bovine Ig monoclonal antibody. After incubation with the culture supernatants, rotavirus was added and subsequently bound antibody was detected using swine anti-rotavirus antibodies conjugated to HRP.

Detection of anti-PMSG bovine antibodies

Screening for monoclonal antibodies directed against PMSG was performed by an indirect double antibody sandwich assay. Microtiter plates were coated with rabbit anti-PMSG antiserum (10 μg Ig/ml, 100 μl/well). After blocking with 1% BSA, a standard solution of PMSG (6 IU/ml) was incubated for 75 min at 37°C. Then culture supernatants were incubated for 1.5 h at rT or overnight at 4°C and rabbit anti-bovine Ig(G,M,A) conjugated to HRP (Nordic Immunological Laboratories) was used as a conjugate. Detailed procedures are similar as for the ELISA described earlier (Booman et al., 1984).

BLV screening

Cell suspensions of the first generation heteromyeloma cell lines were screened for the presence of BLV by a double antibody sandwich assay using monoclonal antibodies directed against two different BLV-p24 antigenic determinants (De Boer et al., 1987). A semi-purified BLV preparation was used as positive control. The heteromyelomas were also tested for the presence of BLV by intraperitoneal inoculation of $5x10^6$ cells in four 6 month-old male Flevolander lambs, of which serum samples were collected in four-weekly intervals over a six months period. The serum samples were tested for anti-BLV antibodies by an agar gel precipitation test and a complex-trapping-blocking ELISA-p24 (De Boer et al., 1987).

Immunofluorescent staining of cell-surface immunoglobulins

The presence of bovine and murine surface immunoglobulins was detected by direct immunofluorescence tests. Cells were incubated (30 min on melting ice) with fluorescein-conjugated rabbit anti-bovine Ig $F(ab')_2$ fragments for the detection of cell-surface bovine Ig and with a rabbit anti-mouse Ig conjugate to detect murine Ig. After washing, fluorescence of cells was examined on a microscope slide under a phase contrast UV microscope.

Karyotyping

Chromosome preparations were made following standard procedures. The chromosomes were examined after application of the GTG-banding technique as described by Hageltorn and Gustavsson (1973). For each cell line to be karyotyped, the total chromosome number and the number of bovine chromosomes were determined on five individual cells. Bovine chromosomes were differentiated from the mouse chromosomes on basis of their negatively stained centromeric regions (Lin et al., 1977).

Characterization of anti-rotavirus and anti-PMSG bovine monoclonal antibodies

To study the reactivity of anti-rotavirus monoclonal antibodies against double-shelled rotavirus of bovine, porcine and/or human origin, one mono-

clonal antibody was further characterized by immunogold electronmicroscopy (Vreeswijk et al., 1988). Briefly, rotavirus was absorbed onto Ni-collodion carbon-coated grids and incubated with monoclonal antibody in PBS for 2 h at 37°C. After washing, bound monoclonal antibody was detected following incubation with 1 μg Protein A/ml PBS (30 min at rT) and subsequently with rabbit anti-Protein A Ig (Sigma), conjugated to colloidal gold (30 min at rT). Some rotavirus-coated grids were floated on a drop of 0.5% glutaraldehyde in PBS for 1 min before the incubation with the monoclonal antibody was carried out. The strains used were bovine rotavirus RA174, purified from a pool of faeces from calves on different farms, porcine rotavirus strain of Pensaert, number 277, probably subtype 1, serotype 4, and finally with human rotavirus, a sample of a child with severe diarrhoea.

The neutralizing capacity of the anti-rotavirus monoclonal antibody was determined by Dr. P.P. Pastoret, Université de Liège, Bruxelles (Thiriart et al., 1987), using bovine rotavirus strain 81/36F (Castrucci et al., 1983).

The cross-reactivity of a bovine anti-PMSG monoclonal antibody with bovine LH and FSH was determined by indirect double antibody sandwich ELISAs (Booman et al., in preparation). The in vitro neutralizing capacity of the monoclonal antibody was determined in a validated assay (M.J.H. Hoeijmakers, Intervet Int. B.V., Boxmeer). Standard solution PMSG was preincubated with the antibody and the mixture was tested for residual PMSG by a double antibody sandwich ELISA. In this assay a mouse monoclonal antibody against PMSG was used for coating and rabbit anti-PMSG-HRP as a conjugate.

Results

Construction and selection of 1st generation heteromyeloma cell lines

Fusion of lymphoid cells from a BLV-infected cow with cells of X63-Ag8.653 resulted in 32 growing hybrid colonies after 6-8 weeks of culture. The hybrid cells contained variable numbers of chromosomes, ranging between 53 and 112 with a mean of 67.6 ± 12.6. The percentage of bovine chromosomes ranged between 0 and 29 with a mean of 14.8 ± 8.4. Only two hybridoma

colonies were positive for bovine cell-surface Ig (1 II and 3 V) and were secreting bovine IgM. Mouse cell-surface Ig was not detected.

On basis of their Ig production, 1 II and 3 V were selected for use as the 1st generation heteromyeloma fusion partners together with 2 hybrids (3 XIV and 1 XXIII) with a high content of bovine chromosomes and favorable growth characteristics. 3 V also contained a high number of bovine chromosomes. No BLV in cell suspensions of 1st generation fusion partners or BLV-specific antibody in serum samples of inoculated lambs could be detected.

Fusion of the 1st generation fusion partners with lymph node cells from calves immunized with rotavirus, resulted in large differences in numbers of hybrid colonies generated by 1 II and 3 V on one hand and 3 XIV and 1 XXIII on the other. The latter two cell lines showed negligible low fusion efficiencies and therefore appeared not to be useful as fusion partners. Although the fusion efficiency of 1 II and 3 V was relatively high (42 and 70, respectively), only 2 hybridomas per fusion partner produced anti-rotavirus antibodies for more than 6 weeks. Three out of these 4 anti-rotavirus antibody-producing cell lines secreted a mixture of IgM and IgG_1 and one cell line secreted only IgG_1. With regard to the total number of hybrid colonies generated after fusion with 1 II and 3 V about 20 and 50%, respectively, produced bovine Ig. About 70% of these bovine Ig-producing hybridomas secreted IgM, 20% IgM + IgG_1 and 10% IgG_1. When karyotyping a group of bovine Ig-producing hybridomas (n = 15), the total number of chromosomes ranged between 68 and 154 with a mean of 111.2 ± 24.9. The percentage of bovine chromosomes varied between 11.6 and 36.5 with a mean of 26.2 ± 4.4.

Comparison of 1st and 2nd generation heteromyeloma cell lines

The hybridoma colonies 13 XIII and 33 VI, generated in fusion experiments with 1 II and 3 V, respectively, were chosen as 2nd generation fusion partners. Both hybridomas secreted anti-rotavirus antibodies for the first 3-4 weeks after fusion, but subsequently lost this activity. They did not secrete unrelated bovine Ig either. In Table 1 characteristics of 13 XIII and 33 VI are compared with characteristics of the 1st generation fusion partners 1 II and 3 V.

In Table 2 results of the fusion experiments of the selected 1st and

TABLE 1

Characteristics of 1st and 2nd generation fusion partners.

fusion partner	number of chromosomes[a] before cloning and aza treatment		number of chromosomes[a] after cloning and aza treatment		cell-surface bovine Ig	(sub)class of secreted bovine Ig	doubling time (h)[b]
	total	bovine(%)	total	bovine(%)			
1st generation							
1 II	58.8	3.8(6.5)	52.3	2.0(3.8)	+	IgM	15
3 V	112.3	27.3(24.3)	98.5	25.5(25.9)	+	IgM	19
2nd generation							
13 XIII	121.2	31.2(25.7)	116.7	21.7(18.6)	-	-	18
33 VI	110.3	30.7(27.8)	113.4	29.4(25.9)	-	-	21

[a]Mean of 5 cells per fusion partner.
[b]2×10^5 cells/10% FCS.

TABLE 2

Fusion results of 1st and 2nd generation fusion partners.

fusion partner	fusion efficiency[a]	spec. anti-rotavirus clones[b]	Ig (sub)class of spec. clones
1st generation			
1 II	151	2	IgM + IgG_1(2)
3 V	332	3	IgM + IgG_1(2), IgG_1(1)
2nd generation			
13 XIII	163	7	IgG_1(5), IgM(2)
33 VI	302	6	IgG_1(3), IgM(3)

[a]Mean number of colonies per 1×10^7 lymphocytes of 2 fusion experiments 3 weeks after fusion.
[b]Total number of specific clones of 2 fusion experiments 6 weeks after fusion.

the 2nd generation fusion partners with lymph node cells from calves immunized against rotavirus are summarized. No difference in fusion efficiency was observed between the first generation heteromyelomas and their second generation derivates. However, the number of specific anti-rotavirus antibody-producing hybridomas was higher with the 2nd generation fusion partners. For this reason and since 13 XIII and 33 VI are non-secretors, these cell lines were preferable to 1 II and 3 V as fusion partners. In the case of the 2nd generation heteromyelomas fusion efficiency of 33 VI was higher than of 13 XIII, but the number of specific antibody-producing hybridomas was about the same. Consequently, subsequent experiments were performed with the two 2nd generation heteromyeloma cell lines.

From 5 antigen-specific antibody-producing hybrid colonies generated from 13 XIII and 33 VI, the total number of chromosomes ranged between 113 to 162 with a mean of 137.8 ± 17.4. The percentage of bovine chromosomes varied between 26 and 31 with a mean of 27.9 ± 1.9. Table 3 summarizes the karyotyping results of hybrid cells after fusion of X63-Ag8.653 with lymphoid cells of the BLV-injected cow and after fusions of bovine lymphocytes with the 1st and 2nd generation fusion partners.

Timing of fusion and source of lymphocytes

Results of fusion experiments of 2nd generation fusion partners 13 XIII and 33 VI with spleen and popliteal lymph node cells on Day 4 or Day 7 after the final intravenous booster injection are summarized in Table 4. It can be concluded that the fusion efficiency of the heteromyeloma cell lines with lymph node cells was considerable lower than with spleen cells; the number of specific antibody-producing hybridomas, however, was much higher with the lymph node cells.

Fusion with lymph node cells on Day 4 resulted in a higher fusion efficiency than fusion on Day 7. For antigen-specific antibody-producing hybridomas, however, fusion on Day 7 was preferable. In contrast, fusion with spleen cells resulted only on Day 4 in specific antibodies, while fusion efficiencies were about equal on Day 4 and 7.

Comparing the fusion partners, 33 VI showed a higher fusion efficiency with lymph node cells than 13 XIII. The number of specific antibody-producing hybridomas generated from 33 VI was, however, lower both with lymph node and spleen cells. The best combination appeared to be fusion of 13

TABLE 3

Chromosomes of bovine-murine hybrid colonies.

number of chromosomes[a]	X63-Ag8.653 x BLV-lymph (bovine-murine hybrids) n=32[b]	bovine x 1st gen. fus. partn. (bovine-[bovine-murine] hybrids) 1 II(n=10)	3 V(n=5)	1 II+3 V(n=15)	bovine x 2nd gen. fus. partn. (bovine-bovine-[bovine-murine] hybrids) 13 XIII + 33 VI(n=5)
total	67.6±12.6	104.4±27.6	124.7±8.4	111.2±24.9	137.8±17.4
bovine	10.8± 7.0	23.3± 8.9	36.8±5.7	29.0± 8.0	38.2± 3.4
% bovine	14.8± 8.4	24.6± 3.8	29.5±3.8	26.2± 4.4	27.9± 1.9

[a]Mean number of chromosomes ± s.d. of hybrid colonies.
[b]Number of hybrid colonies karyotyped.

TABLE 4

Fusion results of 2nd generation fusion partners fused with lymph node cells and splenocytes on Day 4 or 7.

2nd generation fusion partner	fusion efficiency[a]	lymph node cells spec. anti-PMSG clones[b]	number of fusion exp.	fusion efficiency[a]	splenocytes spec. anti-PMSG clones[b]	number of fusion exp.
13 XIII+33 VI						
D(4+7)	54	45	6	99	9	6
D4	62	22	4	104	9	4
D7	37	23	2	90	0	2
13 XIII						
D(4+7)	38	29	3	103	7	3
33 VI						
D(4+7)	69	16	3	95	2	3

[a]Mean number of colonies per 1 x 10^7 lymphocytes of number of fusion experiments 3 weeks after fusion.
[b]Total number of specific clones of number of fusion experiments 6 weeks after fusion.

XIII with lymph node cells on Day 7.

Characterization of specific bovine monoclonal antibodies

Anti-rotavirus antibody

One of the anti-rotavirus antibody-producing hybrid colonies (12 XX), generated by fusion of 1 II with stimulated lymph node cells, was further characterized. The total number of chromosomes before cloning was 120, of which 22 (18%) were of bovine origin. After cloning cells contained 109 chromosomes including 17 (16%) of bovine origin. Before cloning the hybrid cells of 12 XX secreted immunoglobulins of IgM + IgG_1 class, afterwards IgG_1 only. The concentration of bovine IgG_1 in supernatant was about 10 μg/ml per 2.5 x 10^5 cells/48 h. The hybridoma cell line has been actively secreting in culture for more than six months. The doubling time (2 x 10^5 cells, 10% FCS) was about 26 h. In immuno-electronmicroscopy preparations of untreated and glutaraldehyde-treated bovine rotavirus two distinct types of virus particles could be recognized: labelled (Fig. 1A,C) and unlabelled (Fig. 1B) particles. The unlabelled particles were easily identified as single-shelled rotaviruses. Labelled particles in unfixed preparations showed a fuzzy outline (Fig. 1A), while sometimes goldparticles appeared to have drifted away from the characteristic rotavirus

Figs. 1. Colloidal gold immuno-electronmicroscopy carried out directly (Fig. 1A,B) or after fixation (Fig. 1C) on virus particles of bovine rotavirus. Single-shelled virus particles did not show a reaction with the monoclonal antibody (Fig. 1B, ss). After application of a fixation procedure before immunoreactions were carried out, the labelled particles could easily be identified as double-shelled particles (Fig. 1C, ds; arrow, outer shell). Similar results were obtained with swine and human rotaviruses. Bar in (A) indicates 50 nm.

backbone (Fig. 1A, arrowheads). After the application of glutaraldehyde fixation we found gold particles bound to the intact outer shell of double-shelled particles (Fig. 1C, ds; arrow, outer shell). Similar results were obtained with swine and human rotavirus (not shown). In the in vitro neutralization assay according to Thiriart et al. (1987) the antibody did not neutralize bovine rotavirus (strain 81/36 F).

Anti-PMSG antibody

4 XII is a hybrid cell line generated by fusion of 33 VI with stimulated lymph node cells. The total number of chromosomes after cloning was 156, of which 42 (27%) were of bovine origin (no data are available of chromosome content before cloning). The cells of hybridoma cell line 4 XII secreted antibodies of the IgG_1 subclass at a level of 10 - 15 μg per 2.5 x 10^5 cells/48 h. The cell line has been actively secreting in culture for more than six months. Doubling time was about 29 h. The antibodies did not show cross-reactivity with bovine FSH and LH. The in vitro neutralizing capacity of this antibody, present in 10 times concentrated serum-free supernatant (2.5 x 10^5 cells/48 h), was 550 IU PMSG/ml. Cells were easily adapted to growth in serum-free medium (HB 102; HANA Media).

Discussion

Fusion of lymph node cells of a BLV-infected cow with murine myeloma cells resulted in hybrid colonies with a high variability in number of bovine chromosomes. From the observation that two hybrid cell lines retained cell-surface Ig from the parent BLV-cells and secreted bovine IgM, it was reasoned that they would be able to support stable bovine antibody production after fusion with stimulated bovine B-lymphocytes. Although the number of cell lines secreting antigen-specific antibodies was low, this expectation has been confirmed. Whether two additional non-secreting hybrid cell lines, that had been selected as fusion partners only based on a relatively high number of bovine chromosomes and favorable growth characteristics, would also sustain secretion of immunoglobulins, could not be evaluated, as the fusion efficiency of both latter heteromyelomas was rather negligible. The results of the karyotypic analysis of the four first generation heteromyelomas suggest that there is no direct relation-

ship between the number of bovine chromosomes and characteristics important for the selection of the first generation fusion partners. This was likewise reported by Murphy et al. (1986) in human monoclonal antibody production. The few bovine chromosomes present in the cells of heteromyeloma 1 II are apparently sufficient to support endogenous Ig production as well as the generation of stable hybridomas that secrete relatively high yields of bovine Ig. Yarmush et al. (1980) reported that they could not detect any rabbit chromosomes in rabbit-mouse interspecies hybridomas secreting rabbit immunoglobulins and presented evidence for translocation of the relevant rabbit genes to a mouse chromosome. On the other hand, there seems to be a correlation between bovine chromosome content and bovine Ig production, since the higher number of bovine chromosomes present in 3 V compared to 1 II corresponds to the percentages of hybrid Ig-secreting cell lines (50 and 20 percent, respectively). Hybrid cell colonies generated by fusion of the first generation fusion partners retained much more bovine chromosomes than the cell colonies produced by fusion of the mouse myeloma cells with the lymph node cells of the BLV-positive cow.

It was expected that the second generation non-secreting heteromyelomas would still have the apparatus for support of immunoglobulin secretion and would generate a higher yield of antibody-producing cell lines. Indeed, substantially more antigen-specific antibody-producing lines were obtained with the second generation than with the first generation fusion partners. This cannot simply be ascribed to differences in bovine chromosomal numbers (see 3 V and 33 VI and their fusion products). The number of bovine chromosomes in 1 II and 13 XIII was indeed different, but in the fusion products of both heteromyelomas this difference had largely disappeared. For that matter, Tucker et al. (1987), using the same approach, found an increase in the retention of bovine chromosomes in 2nd generation fusion partners compared with 1st generation fusion partners and an increase in number of antigen-specific clones. On average, fusion of the 2nd generation fusion partners with bovine B-lymphocytes resulted in an increase in the absolute number of bovine chromosomes, while the relative number tended to rise to a certain limit. There was considerably less variability in chromosomal number. Apparently, the number of bovine chromosomes has an upper limit. In human-mouse heterohybrids a similar threshold seems to be observed. Hybrids usually have maximally five times more mouse than human chromosomes (Raison et al., 1982; Teng et al., 1985). Whether a third

generation bovine-murine heteromyeloma cell line is beneficial to improve the levels of antibody secretion and to ensure that high levels of secretion are sustained, is currently being explored.

For each of the selected 1st and 2nd generation fusion partners many more antigen-specific immunoglobulin-producing clones could be detected in the first weeks after fusion (data not shown). However, in general their secretion level was very low and within 6 weeks they lost their activity. The numbers of this type of colony were about twice as high as in the mouse-mouse system. A similar limitation occurs in human monoclonal antibody production, generally attributed to either overgrowth by non-secreting lymphocyte 'contaminants' or to the loss of structural genes coding for immunoglobulin. As suggested by James and Bell (1987), other factors include deficits in growth and differentiation factors and their receptors, structural and regulatory gene defects and imperfections in the synthetic and secretory machinery of the cells.

Our experiments indicate that the fusion efficiency is higher with splenocytes than with lymph node cells. However, the number of antigen-specific antibody-secreting clones was lower in the case of splenocytes, notwithstanding the intravenous route for the final booster which preferentially stimulates splenocytes. Apart from our work no comparative studies of this nature have been published in the area of bovine monoclonal antibody production. In contrast to human monoclonal antibody technology, where no differences between spleen and lymph node fusions were found (Jahn et al., 1988), in the mouse fusion of popliteal and inguinal lymph node lymphocytes also induced a higher fusion efficiency as well as a higher frequency of hybridomas secreting specific antibody than fusion with splenocytes (Mirza et al., 1987).

As in murine and human antibody production (for references, see James and Bell, 1987), we found that in cattle the preferred interval between the final booster injection and fusion of splenocytes is 3-4 days. The optimal time for the lymph node cells seems somewhat later. Additional immunocytochemical data from our laboratory (Wissink et al., 1989) show that the appearance of plasma cells in medullary cords of peripheral lymph nodes takes place 4-5½ days after the final booster injection.

Further studies on one of the anti-rotavirus and anti-PMSG monoclonal antibodies proved the usefulness of the heteromyelomas for the production of bovine monoclonal antibodies. Both antibody-producing cell lines showed

good cloning efficiency (about 30%), cells can grow in a minimal medium even under serum-free conditions and secretion levels obtained are relatively high in comparison with other reports in bovine (Tucker et al., 1984; Groves et al., 1987a) as well as in human monoclonal antibody production (for references, see Kozbor and Croce, 1985). In addition, preliminary results from our laboratory indicate that it is possible to obtain bovine monoclonal antibodies via ascites production or high serum titers in irradiated (300 rad), pristane-treated calves by inoculation of at least 1×10^8 hybrid cells (data not shown). Although the anti-rotavirus antibody was directed against an outer capsid glycoprotein, which might theoretically carry antigenic sites for virus neutralization, the antibody did not neutralize bovine rotavirus in vitro. The in vitro neutralizing capacity of the anti-PMSG monoclonal antibody was very potent. Further studies on the in vivo neutralizing capacity of this antibody are currently in process. Since a murine anti-PMSG monoclonal antibody has recently become available commercially (Neutra-PMSG, Intervet), a comparative study of the inhibitory capacity and the efficacy of the bovine and murine antibodies after repeated treatment is now possible.

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CHAPTER 7

A COMPARATIVE STUDY ON THE USE OF BOVINE AND MURINE MONOCLONAL ANTIBODIES FOR PASSIVE IMMUNIZATION IN CATTLE

P. Booman, H.C.G. Wissink, M. de Bruijn, R. Veerhuis and A.M. Hengst

Research Institute for Animal Production "Schoonoord",
P.O. Box 501, 3700 AM Zeist (The Netherlands)

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Abstract

In the present experiments the efficacy of murine and bovine monoclonal antibodies for passive immunization in cattle was compared. The in vivo immunoneutralization of pregnant mare serum gonadotrophin (PMSG) by murine and bovine antibodies after repeated administration was chosen as a model for this study. Results indicate that repeated injections of murine monoclonal antibodies against PMSG (mMCA) alone did not, or only to a small degree, elicit an anti-mouse immune response. The simultaneous administration of mMCA and PMSG resulted in relatively high levels of anti-mouse antibodies after the second injection, leading to a decrease in neutralizing activity of mMCA. The results suggest that the neutralizing activity of mMCA is inhibited more by anti-idiotypic than by anti-isotypic antibodies against mMCA. In vivo, the bovine monoclonal antibody against PMSG (bMCA) only partially neutralizes PMSG. After repeated administration of bMCA, either alone or in combination with PMSG, no anti-bMCA antibodies could be detected in our assay system. In addition, no change in plasma levels of bMCA and PMSG compared with levels after the first injection was observed. Although it has to be confirmed by further experiments whether our findings can be generalized, the present results suggest that for repeated passive immunization in cattle homologous antibodies are to be preferred above heterologous antibodies.

Introduction

In animal production monoclonal antibodies are increasingly finding application in the area of passive immunization, for example in the immunomodulation of reproductive processes (Booman, 1988). A limiting factor for the use of monoclonal antibodies in passive immunization when applied in other species might be their murine or rat origin. Human clinical trials with murine monoclonal antibodies indicate that almost all non-immunocompromised patients develop anti-mouse immunoglobulin antibodies which inhibit the therapeutic effect (Cole et al., 1985). In pigs, for instance, a significant antibody response to mouse immunoglobulins is generated after repeated administration of murine antibodies (Booman et al., 1989a). The immune response may be directed partially against the isotypic determi-

nants, partially against the idiotypic determinants of the antibodies administered (Schroff et al., 1985; Shawler et al., 1985). In addition, heterologous immunoglobulins lack species-specific effector determinants which are necessary for cooperative cellular effects (Nose and Wigzell, 1983).

Although anti-idiotypic responses cannot be excluded as a complicating factor, homologous antibodies might offer some advantages over their murine counterparts in terms of effectiveness for passive immunization. At our laboratory a bovine-murine heteromyeloma cell line for the production of bovine monoclonal antibodies has been constructed (Booman et al., 1989b), which offers the possibility to compare the efficacy of bovine and murine antibodies after repeated administration to cattle. The neutralization of simultaneously administered pregnant mare serum gonadotrophin (PMSG) was chosen as a model for such a study. PMSG is used in cattle embryo transfer programmes to induce superovulation. The prolonged presence of the exogenous gonadotrophin may cause adverse effects, leading to a relatively high percentage of embryos of poor quality. To prevent the prolonged action of PMSG, antibodies against the gonadotrophin are administered to superovulated cattle immediately after insemination. These antibodies neutralize the residual PMSG in circulation and, consequently, the number of embryos of good quality may increase (Dieleman et al., 1989).

Materials and Methods

Animals

Twelve Holstein Friesian x Dutch Friesian heifers with normal oestrous cycles were kept on a slatted floor in a loose housing system. All animals were 14 months old and weighed ± 300 kg at the beginning of the experiments. Before the experiments started, oestrus was synchronized by Syncro Mate-B (Intervet). The day an animal came into oestrus was defined as D_0.

Antibodies

Neutra-PMSG (Intervet), a commercially available monoclonal antibody preparation against PMSG, served as a source for murine monoclonal antibodies (mMCA). One vial of Neutra-PMSG contained 5 ml of solution with

a concentration of 200 μg IgG_1/ml, which neutralizes residual circulating PMSG in cows, 3 to 5 days after administration of up to 3000 IU PMSG.

Bovine monoclonal antibodies against PMSG (bMCA) were produced by hybridoma cell line 4 XII (IgG_1), obtained by fusion of bovine-murine heteromyeloma cells with stimulated lymph node cells of calves (Booman et al., 1989b). Cells were cultured on serum-free cell culture medium (HB 102; HANA Media). The supernatant was concentrated tenfold on an XM-50 Diaflo membrane (Amicon Corp.). Two different batches of culture supernatant with a concentration of 80 and 120 μg IgG_1/ml were used for in vivo neutralization experiments. The in vitro neutralizing capacities of both batches of bMCA, determined by procedures described below, were 4.8 and 4.2 IU PMSG/μg, respectively.

As a control a bovine polyclonal antiserum against PMSG (bPCA) was produced by injecting 2 heifers intramuscularly with 3000 IU PMSG (Folligonan; Intervet), emulsified in complete (first injection) or incomplete Freund's adjuvant at 5 four-weekly intervals. Heifers were boosted intravenously with 5000 IU PMSG in PBS 8 weeks after the last injection and were slaughtered four days later. For serum production blood was allowed to clot at 37°C for one h and subsequently at 4°C for 6 h. The serum, separated by centrifugation at 1600g for 10 min at 4°C, was pooled and stored at -80°C. The in vitro neutralizing capacity of the bPCA was 1000 IU PMSG/ml.

In the present experiments heifers were injected with 3000 IU PMSG. The amount of mMCA per injection was 5 ml of Neutra-PMSG (1 mg IgG_1). The neutralizing capacity of Neutra-PMSG is lower in vivo than in vitro (data Intervet). As this might also be the case for bMCA, the amount of bMCA used in the in vivo experiments was determined as the quantity that could neutralize 6000 IU PMSG in vitro (6 ml of bPCA). However, because of incorrect determination of the in vitro neutralization capacity of bMCA, in vivo experiments with bMCA were in fact performed with 1 or 1.8 mg of bMCA, estimated to neutralize 4200 and 8550 IU PMSG, respectively.

None of the antibodies used in this study did cross-react with bovine FSH and LH in vitro, as determined by the procedures described below.

Experimental design and blood sampling

Six groups of two heifers were treated on Day 11 ± 1 of the first oestrous cycle after synchronization according to the following scheme:

mMCA group: injected with murine monoclonal antibody (heifers 12 and 13);
mMCA+PMSG group: injected with mMCA plus 3000 IU PMSG (heifers 2 and 6);
bMCA group: injected with bovine monoclonal antibody (heifers 26 and 29);
bMCA+PMSG group: injected with bMCA plus 3000 IU PMSG (heifers 25 and 28);
bPCA group: injected with bovine polyclonal antiserum (heifers 14 and 15);
bPCA+PMSG group: injected with bPCA plus 3000 IU PMSG (heifers 16 and 23).
Fourty-eight h after administration of the antibodies (and PMSG) each animal received 2 ml of a prostaglandin F2α analogue (Prosolvin; Intervet) in order to induce oestrus 48-72 h later. Between D_9 and D_{13} of the second cycle the number of ovulations was determined by rectal palpation of the ovaries. On D_{16} of the second cycle each animal received another 2 ml of Prosolvin to induce the third oestrus. The period between the very first D_0 and the third D_0 was defined as one complete experimental period. A second and third experimental period followed according to the same treatment schedules for the respective groups. The interval between injections was about 35 days.

The first time animals from the bMCA group and heifer 25 from the bMCA + PMSG group were injected with the larger dose of bMCA (1.8 mg). Antibodies were injected intravenously into the jugular vein. PMSG and Prosolvin were injected intramuscularly.

Blood samples were collected from the heifers treated with mMCA and bMCA alone or in combination with PMSG each day of the experimental periods between 6.00 and 8.00 h. On the day of injection with antibodies (plus PMSG) samples were also collected immediately after injection at 9.00 h and subsequently at 11.00, 17.00 and 21.00 h. On the first two days after injection a blood sample was taken between 16.00 and 18.00 h, in addition to the daily sample.

Blood samples were collected from the jugular vein in heparinized vacutainer tubes (Venoject; Terumo) and chilled immediately on ice. Plasma, separated by centrifugation at 1600g for 10 min at 4°C, was stored at -20°C.

When oestrus was not observed, levels of progesterone were checked in selected plasma samples in order to test reproductive activity.

Enzyme immunoassays

For all enzyme immunoassays described below, detailed assay procedures were similar to those described earlier (Booman et al., 1984), unless mentioned otherwise.

Determination of in vitro neutralization of PMSG

The in vitro neutralizing capacity of the monoclonal antibody preparations was determined in a validated assay by Intervet Int. (The Netherlands). Equal quantities of standard solution of PMSG (0-4 IU/ml) and antibodies or control serum in different dilutions were preincubated at 4°C and the mixture was tested for residual PMSG in a double antibody sandwich enzyme immunoassay. In this assay a mouse monoclonal antibody against PMSG (Intervet) was used for coating and rabbit anti-PMSG coupled to horseradish peroxidase (HRP) as a conjugate.

Cross-reactivity with bovine LH and FSH

Cross-reactivity of antibody preparations with bovine LH and FSH was determined in indirect double antibody sandwich assays. For the LH assay, microtiter plates were coated with rabbit anti-bovine LH (bLH) (10 μg Ig/ml, 100 μl/well). After blocking with 1% BSA, wells were incubated with 10 ng bLH for 75 min at 37°C. The antibody preparations were incubated for 1.5 h at rT and rabbit anti-bovine Ig(G,M,A)-HRP or rabbit anti-mouse Ig-HRP (Nordic Immunological Laboratories) were used as conjugates. Characterization of rabbit anti-bLH and bLH has been described by Dieleman et al. (1983). The assay for FSH was similar to that for LH, except that rabbit anti-bFSH (UCB Bioproducts; Belgium) was used as the first antibody and bFSH (bFSH-B-1; USDA Animal Hormone Program) instead of bLH.

Circulating antibody capable of binding PMSG

In order to monitor the levels of circulating antibody still capable of binding PMSG an indirect double antibody sandwich assay was performed. Microtiter plates were coated with rabbit anti-PMSG antiserum (10 μg Ig/ml, 100 μl/well). After blocking with 1% BSA, a standard solution of PMSG (6 IU/ml) was incubated for 1.5 h at 37°C. Then plasma samples were incubated for 2 h at rT or overnight at 4°C and rabbit anti-mouse Ig-HRP was used as a conjugate to determine levels of mMCA and rabbit anti-bovine Ig-HRP

for levels of bMCA.

Significant increases in plasma levels of mMCA and bMCA were defined as all values higher than the average background value plus twice the standard deviation of all samples from the same heifer taken before first injection.

Circulating PMSG able to be bound by antibody

The levels of PMSG still able to be bound by antibody were monitored by coating microtiter plates with the respective mMCA and bMCA (10 μg IgG_1/ml, 100 μl/well). After blocking, plasma samples were incubated for 2 h at rT or overnight at 4°C and bound PMSG was detected using rabbit anti-PMSG antibodies conjugated to HRP. Significant increases in PMSG levels were defined as for mMCA and bMCA.

Determination and quantification of anti-mMCA and anti-bMCA immune response

bMCA were purified by ammonium sulphate precipitation, followed by gel filtration on a Sephacel HS-200 column (Pharmacia). Purity and molecular weight were checked by SDS-PAGE (Laemmli, 1970) or HPLC. mMCA and an irrelevant mouse monoclonal antibody directed against human chorionic gonadotrophin (hCG), with the same isotype (IgG_1) and the same type of light chain (κ), were kindly provided in purified form by Intervet and Diosynth (The Netherlands), respectively. Biotinylation of purified antibodies was performed as described by Wagener et al. (1984). Antibody preparations were stored at -80°C in 50% glycerin.

For determination of anti-mMCA antibodies, purified mMCA or anti-hCG (2 μg/ml; 100 μl/well) were absorbed onto microtiter plates. After blocking with 0.5% gelatin in PBS for 30 min at 37°C, plasma samples of heifers treated with mMCA (either with or without PMSG) were incubated in mMCA- and anti-hCG-coated plates for 1 h at 37°C. Affinity-purified rabbit anti-mouse IgG_1 antibodies (Miles) in preimmune bovine plasma (5000 - 20 ng/ml) were included in each plate as a standard.

For determination of anti-bMCA antibodies, plasma samples of animals treated with bMCA (either with or without PMSG) were diluted in a buffer containing a high percentage of salt (0.5M NaCl, 15mM $Na_3PO_4.12H_2O$, 0.05% w/v Tween 80, pH 7.2) and incubated in bMCA-coated wells (5 μg/ml; 100 μl/well). In this assay, serial dilutions of protein A-purified rabbit

anti-bovine IgG_1 (Nordic Immunological Laboratories) in PBS (40 - 2 µg/ml), supplemented with 20 mg rabbit serum Ig/ml and 33 mg ovalbumin/ml, were used as a standard. Both anti-mMCA and anti-bMCA plates were subsequently incubated (1 h at 37°C) with the appropriate biotinylated antibodies diluted 1:1600 (mMCA and anti-hCG) or 1:800 (bMCA) in assay buffer (0.04M PBS, 0.1% BSA). Finally, bound biotinylated antibodies were detected by incubation with streptavidin-HRP (Amersham), diluted 1:5000 in assay buffer with 1% porcine serum, for 45 min at rT. Linear regression was used to quantify anti-MCA antibodies in plasma samples. The detection limits of the assays were 50 ng/ml (mMCA) and 500 ng/ml (anti-hCG and bMCA).

Other assays

Determination and quantification of bovine Ig-isotypes was performed by a double antibody sandwich assay as described by Van Zaane and IJzerman (1984).

Progesterone levels in plasma samples were determined by a competitive assay (Van de Wiel and Koops, 1986).

Results

In vivo neutralizing capacity of anti-PMSG antibodies

The in vivo neutralizing capacity of the anti-PMSG antibodies was monitored by rectal palpation of the ovaries of the injected heifers. Relevant results are presented in Table 1. No influence on the number of ovulations, counted as corpora lutea (CL), was observed in the heifers treated with the antibodies only. One CL could be detected in 5 out of 6 animals in nearly all experimental periods. Heifer 29 from the bMCA group differed in that no CL or follicle was felt during palpation after the first treatment. Exceptionally, in mMCA-treated heifer 12 two CL were present continuously.

The simultaneous administration of antibody and PMSG resulted in an increase of the number of ovulations as treatment proceeded in the heifers from the mMCA + PMSG group. One CL could consequently be detected in the two bPCA plus PMSG-treated heifers. In heifers of the bMCA + PMSG group highly stimulated ovaries were found in 3 out of 6 cases. It appeared that

the in vivo neutralizing capacity of bPCA remained unchanged, whereas the efficacy of mMCA decreased after repeated administration. Finally, bMCA were not able to inhibit PMSG-activity in vivo in all cases.

Levels of mMCA and bMCA capable of binding PMSG

Levels of mMCA capable of binding PMSG in plasma of mMCA-treated heifer 12 were increased significantly after each injection (Fig. 1). In heifer 13 from the mMCA group, the increase in mMCA level was less obvious after the second injection and almost absent after the third (Table 1). Fig. 1 shows that on some occasions antibody levels were elevated in between the injections.

Compared to the mMCA group, injection with mMCA plus PMSG resulted in a less pronounced increase in mMCA levels after the first injection. The observed increase in mMCA levels directly after injection was partly abolished after repeated treatment (Fig. 2; Table 1).

When bMCA were administered, a small increase in plasma levels of bMCA capable of binding PMSG could be observed after each injection. In animals from the bMCA + PMSG group the levels of bMCA were elevated only after the second injection in heifer 28 (Table 1). As with mMCA levels in the mMCA group, bMCA levels were sometimes elevated in between the injections (Fig. 3). Similar patterns of plasma MCA levels were observed in pigs injected with different amounts of mMCA (unpublished results).

Levels of PMSG able to be bound by antibody

Plasma levels of PMSG able to be bound by antibody in heifers from the mMCA + PMSG group remained at the baseline after the first injection (Fig. 2). Distinct PMSG peaks were, however, observed after the second and third injection (heifer 6; Fig. 2) or after the third injection only (heifer 2; Table 1).

When animals were injected with bMCA + PMSG, a similar, although moderate, elevation of PMSG levels was observed after each injection in heifer 25 (Fig. 3), whereas in heifer 28 PMSG levels were moderately increased after the first injection and very strongly after the second one. After the third injection a very slight increase was observed in this heifer (Table 1).

TABLE 1

Effect of administration of mMCA and bMCA either alone or together with PMSG on plasma levels of mMCA and bMCA capable of binding PMSG, PMSG able to be bound by antibody and of anti-mMCA and anti-bMCA antibodies and on number of corpora lutea in different experimental periods.

Group		mMCA		mMCA + PMSG		bMCA		bMCA + PMSG	
No. heifer		12	13	2	6	26	29	25	28
mMCA/bMCA[a]	period 1	++	++	+	+	+	+	-	-
	2	++	+	+	+/-	+	+	-	+
	3	++	+/-	-	-	+	+	-	-
PMSG[a]	period 1			-	-			+	+
	2			-	++			+	++
	3			+	++			+	+/-
anti-mMCA/bMCA[a]	period 1	-	-	-	-	-	-	-	-
	2	-	+/-	++	++[c]	-	-	-	-
	3	-	+	++	++	-	-	-	-
corpora lutea[b]	period 1	2	1	1	2	1	0[d]	1	*[e]
	2	2	2	1	5	1	1	*[e]	*[e]
	3	2	1	2	4	1	1	1	1

[a]Semi-quantitative presentation of plasma levels of mMCA/bMCA, PMSG and anti-mMCA/bMCA: ++ relatively large increase; + a small, but significant ($\geq \bar{x}$ + 2 s.d.) increase; +/- a very small, but still significant increase; - no significant increase.
[b]Number of corpora lutea determined by rectal palpation of the ovaries; in all animals of the bPCA and bPCA + PMSG groups each experimental period one corpus luteum was observed.
[c]Largely anti-idiotypic.
[d]No corpus luteum or follicle was felt during palpation; based on plasma progesterone levels reproductively active.
[e]Large ovaries with many corpora lutea.

Anti-mMCA and anti-bMCA immune response

Repeated administration of mMCA alone (mMCA group) did not induce high bovine anti-mouse titers. In heifer 12 no anti-mMCA response could be detected at all; in heifer 13 a slight immune response against the mMCA was detectable after the second injection (102 ng/ml plasma). After the third injection the anti-mMCA antibodies reached a level of 567 ng/ml plasma

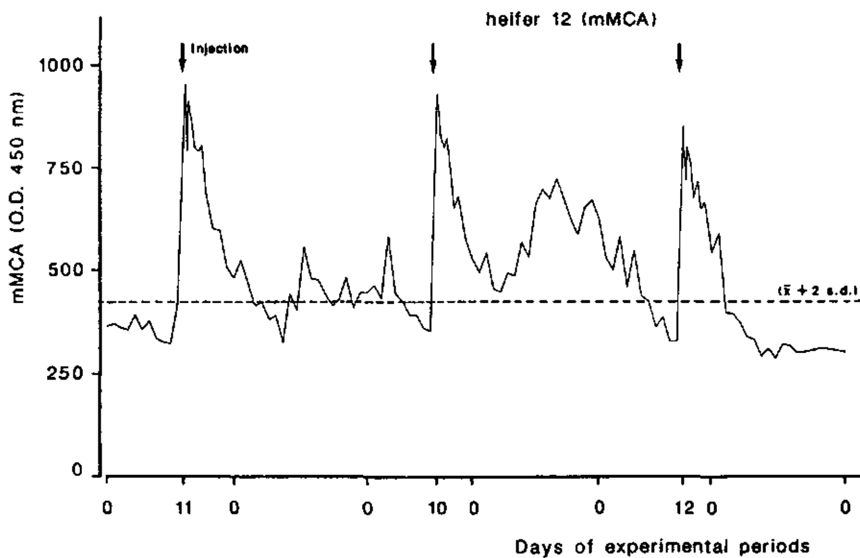

Fig. 1. Plasma levels of mMCA capable of binding PMSG after repeated injection of mMCA.

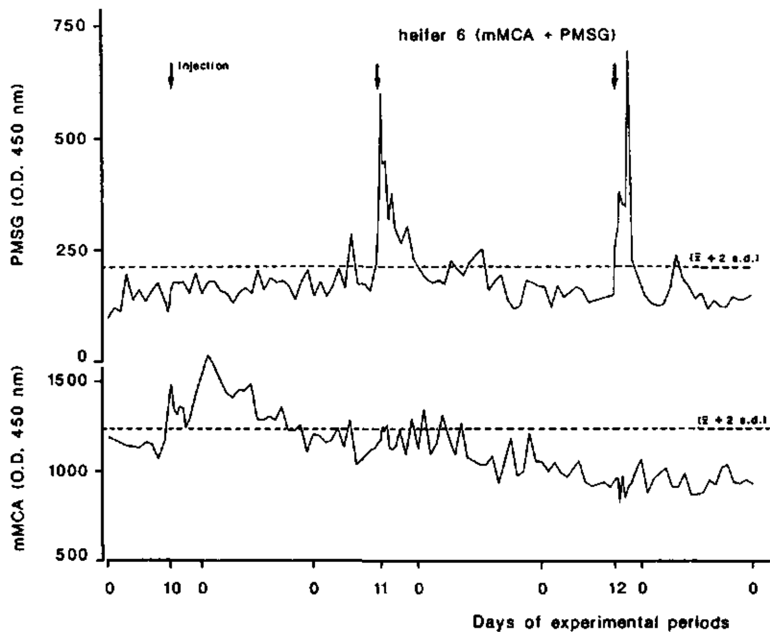

Fig. 2. Plasma levels of mMCA capable of binding PMSG and of PMSG able to be bound by antibody after repeated injection of mMCA and PMSG.

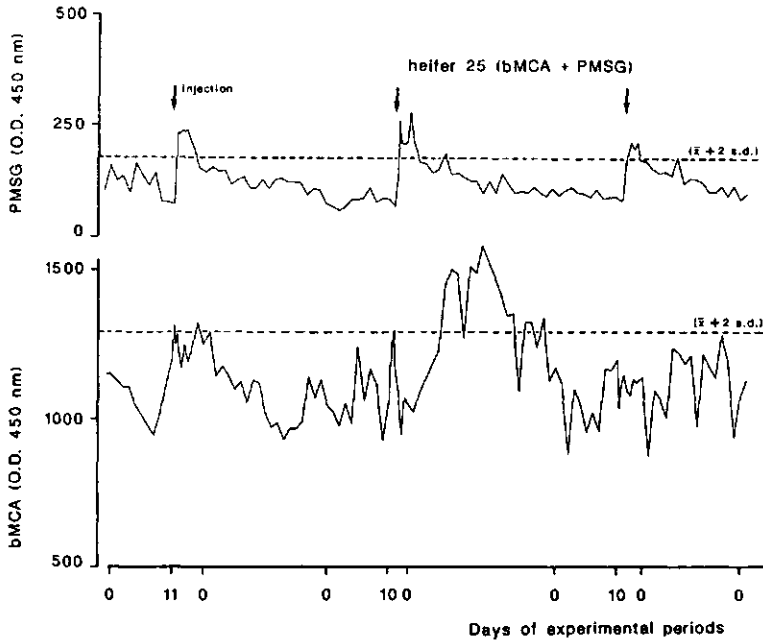

Fig. 3. Plasma levels of bMCA capable of binding PMSG and of PMSG able to be bound by antibody after repeated injection of bMCA and PMSG.

in this heifer. To discriminate between anti-isotypic and anti-idiotypic antibodies, the presence of anti-isotypic (mouse $IgG_1; \kappa$) antibodies in plasma samples was tested in an enzyme immunoassay in which the wells were coated with an irrelevant monoclonal antibody. No binding to the irrelevant mouse monoclonal antibody was detectable.

The humoral response of heifers from the mMCA + PMSG group was characterized by a relatively strong increase in anti-mMCA antibodies shortly after the second injection and a further increase after the third administration (Figs. 4 and 5). In heifer 6 (Fig. 5) a temporary drop in antibody titer occurred immediately after the last administration of mMCA plus PMSG. In the insert in Fig. 5 this phenomenon is shown in more detail. The nature of the anti-mouse response was largely anti-isotypic, except in heifer 6 after the second injection, when a predominantly anti-idiotypic response was observed (Fig. 5).

In the animals of the bMCA and bMCA + PMSG groups no anti-bMCA antibodies could be detected at all.

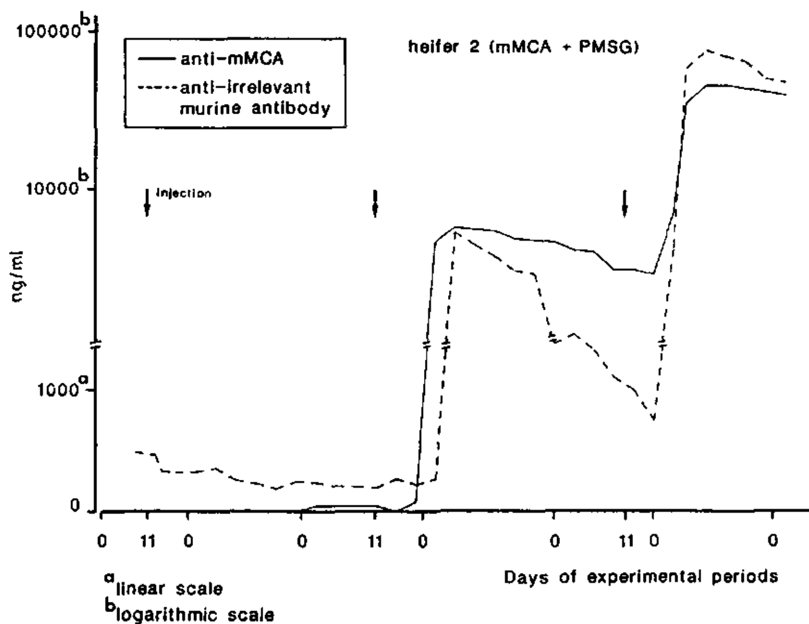

Fig. 4. Plasma levels of anti-irrelevant murine antibody and of anti-mMCA antibodies after repeated injection of mMCA and PMSG.

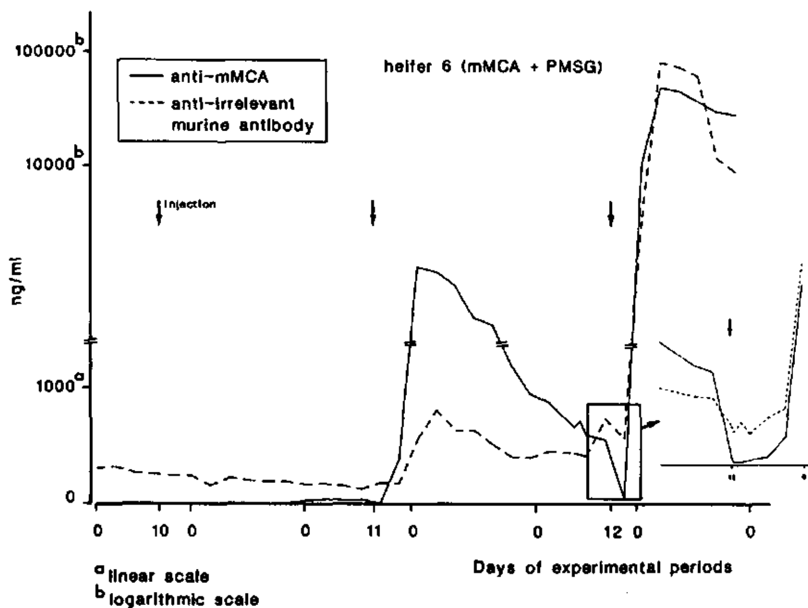

Fig. 5. Plasma levels of anti-irrelevant murine antibody and of anti-mMCA antibodies after repeated injection of mMCA and PMSG.

Discussion

When monoclonal antibodies are to be administered repeatedly, e.g. for therapeutic reasons or in order to influence physiological processes, the use of homologous rather than heterologous monoclonal antibodies may be preferred, since a humoral response against the injected xenogeneic proteins may neutralize their physiological effect. The use of homologous monoclonal antibodies may, however, still elicit an anti-idiotypic response. When murine monoclonal antibodies are used for tumor therapy in man, such an anti-idiotypic response may represent a substantial part of the anti-mouse antibodies present in circulation (Herlyn et al., 1986; Chatenoud et al., 1986; Traub et al., 1988). It is generally assumed that anti-idiotypic antibodies are of major clinical importance, since only they may abrogate the therapeutic effectiveness of the injected antibody (Baudrihaye et al., 1984; Chatenoud et al., 1986).

It can be questioned, however, whether the reported anti-idiotypic response observed when heterologous monoclonal antibodies are applied will occur to a similar extent when homologous antibodies are administered, since the formation of the anti-idiotypic antibodies may be enhanced by a hapten-carrier system, in which the heterologous immunoglobulin acts as a carrier for the idiotype, a mechanism suggested by Ehrlich et al. (1987). Their findings, that multiple injections of moderate amounts of human monoclonal antibodies in rhesus monkeys resulted in an anti-idiotypic response in only 1 out of 5 animals (Ehrlich et al., 1987), and that repeated administration of large doses (16 mg/kg) of human monoclonal antibodies to rhesus monkeys failed to result in an anti-human response (Ehrlich et al., 1988), suggest that under the conditions usually employed (i.e., intravenous injection and low idiotype concentration) the idiotype may not be very immunogenic if the proposed carrier effect is absent. Large scale studies on the immunogenicity of human monoclonal antibodies in man have not been performed thus far, however, leaving the question unanswered whether repeated administration of homologous antibodies offers advantages compared with heterologous antibodies.

The ability to produce bovine monoclonal antibodies in our laboratory enabled us to address this question in the bovine system. As a model the biological effectiveness and the immunogenic potential of anti-PMSG bovine and murine monoclonal antibodies (bMCA and mMCA, respectively) were com-

pared in cattle. In order to determine the effectiveness of the antibodies administered, the in vivo PMSG-neutralizing effect was monitored in heifers by rectal palpation of the ovaries. As a control 2 groups of animals were injected with a bovine anti-PMSG polyclonal antiserum (bPCA), either alone or together with PMSG. One CL could be detected consistently in all heifers after repeated injections, indicating that the ovaries functioned normally and that repeated treatment did not change the efficacy of the bPCA. Moreover, when mMCA or bMCA alone were administered, no change in the number of CL was observed (Table 1), indicating that the experimental design did not influence the reproductive processes.

In one of the two heifers injected with mMCA a more pronounced suppression in the rise of circulating mMCA levels was observed after the second and in particular after the third injection. This decrease in mMCA levels coincided with an increase in levels of anti-mouse antibodies (heifer 13; Table 1). In the other heifer injected with mMCA no such effect was observed (heifer 12; Table 1). Whether the anti-mouse response was directed against the idiotypic part or against the isotypic part of the mMCA could not be clarified because of the relatively high detection limit of the appropriate assay.

Repeated administration of bMCA did not affect levels of bMCA or result in a detectable anti-bMCA response (heifers 26 and 29; Table 1). It should be noted, however, that the detection limit of the anti-bMCA assay is 500 ng Ig/ml plasma, whereas the amount of anti-mMCA antibodies in mMCA-treated heifer 13 was not more than 567 ng Ig/ml plasma.

When bMCA or mMCA were simultaneously administered with an intramuscular injection of PMSG, a gradual increase in number of CL was observed in the mMCA plus PMSG injected animals (Table 1). The observed decrease in in vivo neutralizing capacity of mMCA is in agreement with the increased levels of circulating PMSG detected in the enzyme immunoassay (Fig. 2; Table 1). These findings correlate with the rapid clearance of the mMCA from circulation after the second and third injection (Table 1). This clearance may be caused by the with time increasing anti-mouse response which was largely anti-idiotypic in one of two heifers after the second injection (Figs. 4 and 5). When injected intramuscularly, PMSG reaches its maximum plasma concentration 12 to 24 h after injection (Menzer and Schams, 1979). Therefore, a considerable part of the intravenously administered mMCA can be bound by anti-mMCA antibodies before it may form a

complex with PMSG. This may explain the temporary drop in anti-mMCA antibody levels (insert Fig. 5), that occurred immediately after the last treatment.

The different reactions observed in the mMCA plus PMSG treated animals after the second injection (heifer 6: largely anti-idiotypic, increased PMSG levels and stimulated ovaries; heifer 2: anti-isotypic antibodies, baseline levels of PMSG and non-stimulated ovaries; Table 1) suggest that mMCA are hindered in their PMSG-neutralizing activity more by anti-idiotypic than by anti-isotypic antibodies. When present in larger quantities, anti-isotypic antibodies can also prevent the neutralizing activity of mMCA, as is illustrated by the presence of high PMSG levels and an increased number of CL in both heifers after the third injection with mMCA plus PMSG, when anti-isotypic antibodies are dominantly present. This is not completely in line with the currently accepted idea that only anti-idiotypic antibodies can block monoclonal antibody functions (Chatenoud, 1986). We cannot exclude, however, the possibility that there is a small anti-idiotypic component in the anti-mouse response that is below the "cut-off" level in our assay.

Typically, when mMCA were injected together with PMSG, a more dramatic humoral response against the mMCA was observed than when mMCA alone were administered (Table 1). A possible explanation for this phenomenon may be that, due to their larger molecular size, the PMSG - anti-PMSG immune complexes formed in vivo are more rapidly cleared from circulation than monomeric mouse IgG_1. As a consequence, levels of processed immune complexes exposed on antigen-presenting cells exceed that of processed monomeric IgG_1, the latter probably remaining below threshold levels necessary for triggering of an immune response. Moreover, it should be noted that both PMSG and mMCA are xenogeneic proteins.

No increase in bMCA levels in plasma from bMCA plus PMSG-treated animals was found, except in heifer 28 after the second injection. This rise in the level of circulating bMCA coincides with a strong rise in PMSG levels (Table 1). Although levels of bMCA remained low after injection of bMCA plus PMSG, no anti-bMCA antibodies could be detected. Several other types of assays were employed (e.g. an inhibition assay, Wissink et al., 1989), but these also failed to detect anti-bMCA antibodies. These findings are in agreement with the observations that in the bMCA plus PMSG-treated heifers the PMSG plasma levels are in general very regular in height after

the successive injections and no clear relationship exists between the number of ovulations and the experimental periods (Table 1).

Apparently bMCA were not able to prevent multiple ovulation in all cases. Although a direct relationship between bMCA and PMSG levels indicates that binding of bMCA to PMSG occurs in vivo, PMSG levels in plasma from bMCA plus PMSG-treated animals were found to be slightly, but significantly, increased to values in between the baseline levels obtained with mMCA plus PMSG-treated heifers and the much higher levels observed in a control animal injected with 3000 IU PMSG only (not shown). These data, together with the finding that bMCA levels did not rise after injection of animals with bMCA plus PMSG, may suggest that higher doses of bMCA have to be injected to neutralize PMSG activity in vivo.

Data from an additional experiment indicate that bMCA in the dosages used only partially suppress multiple ovulation, i.e. partially neutralize the activity of PMSG in vivo (De Bruijn and Booman, 1989). In this experiment two groups of 4 cows were injected on Day 11 ± 1 of the cycle with 3000 IU PMSG in combination with either 1.4 or 2.1 mg bMCA, capable of neutralizing 6000 and 9000 IU PMSG in vitro, respectively. A control group of 15 cows was treated with PMSG only. The number of CL (means ± standard deviations) were 2.5 ± 1.5 and 5.0 ± 3.8 for the groups treated with 1.4 and 2.1 mg bMCA, respectively, compared with 10.7 ± 2.1 for the control group.

When the different PMSG-neutralizing capacities of mMCA and bMCA in vivo are taken into account, together with the possible differences in association constants of mMCA and bMCA for PMSG, the restricted number of animals tested and the limitations of the assays, one may conclude that the efficacy of the murine antibodies decreases due to the anti-mouse antibody response after repeated administration, whereas no such effect was found for the bovine antibodies. These results support the carrier-hapten effect hypothesis proposed by Ehrlich et al. (1987). Although our results indicate that in cattle homologous antibodies might offer advantages over their murine counterparts for repeated passive immunization, it has been shown that murine monoclonal antibodies can be succesfully applied in cattle treated for superovulation (Dieleman et al., 1989). Moreover, in cows that were treated repeatedly for superovulation the efficacy of the murine antibody could be demonstrated (Gielen et al., 1989). We are currently extending our experiments to confirm the generality of our findings.

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SUMMARY AND FINAL REMARKS

One of the landmarks in immunology was the invention and development of monoclonal antibody-secreting hybridomas by Milstein and his coworkers. The enormous promise of monoclonal antibody technology, which became apparent soon after its discovery, may explain the unusual speed with which monoclonal antibodies have been applied to biological and medical sciences.

In animal production monoclonal antibodies are increasingly finding application in the areas of diagnostics, passive immunization and fundamental research. In Chapter 1 of this thesis some applications of monoclonal antibodies within these areas, with emphasis on reproduction, are discussed. It has been concluded that the particular advantages of monoclonal antibodies can firstly and most easily be shown in immunodiagnosis. Once a hybridoma producing a monoclonal antibody appropriate for a particular application has been obtained, large amounts of a homogeneous and reliable reagent are available for as long as they are needed. As ingredients in test kits, monoclonal antibodies have rapidly replaced conventional polyclonal antibodies.

An example of a typical diagnostic test in animal production in which monoclonal antibodies might be used is the milk-progesterone test for confirmation of oestrus and pregnancy diagnosis in cattle. In Chapter 2 the production and characterization of monoclonal antibodies against progesterone have been described in order to standardize an enzyme immunoassay for milk-progesterone. The antibodies differed considerably in their binding affinity for progesterone and showed distinct specificities for a variety of steroids. Results show that, although the technique of monoclonal antibody production selects antibodies specific for a single antigenic determinant, this does not always preclude the possibility of cross-reactivity. Moreover, most antibodies produced have affinities far below the corresponding conventional antisera, as has been discussed in Chapter 1. The monoclonal antibody with the highest association constant and relatively good specificity did not detect progesterone with any greater sensitivity than the conventional polyclonal sera. However, since monoclonal antibodies avoid the dependency upon animals producing high quality antisera and improve test standardization, a commercially available rapid progesterone cow-side test has been designed based on the monoclonal antibody with the best characteristics.

Results of Chapter 2 underline the necessity to evaluate carefully the need of producing monoclonal instead of polyclonal antibodies for a given antigen, as considerable time and effort are required to obtain a monoclonal antibody with suitable properties. Preselection of antibodies on affinity and specificity in an early stage makes the monoclonal antibody technology more efficacious. In our laboratory a cocktail of related steroids was used to enable such an early selection of antibodies against either oestrone or oestrone sulphate. Testing the replacement of oestrone, coupled to horse-radish peroxidase, from the antibodies made it possible to produce high affinity monoclonal antibodies with nearly unique specificities in a relatively short time. Experiments are in progress to develop a test for pregnancy diagnosis in pigs in faecal samples based on those antibodies.

Another application in diagnostics is the use of monoclonal antibodies against a male-specific protein, the H-Y antigen, for sexing bovine embryos before implantation. H-Y is a weak antigen and immunization with H-Y antigen in an inbred strain of mice usually results in production of low titered, low affinity antisera. Because only a low percentage of mice has a good antibody response and their sera run out quickly, monoclonal antibodies were produced (Chapter 3). Male specificity of the antibodies was tested in a variety of assays including enzyme immunoassays based on various sources of soluble H-Y and indirect immunofluorescence assays based on binding of the antibodies to H-Y antigen on the cell surface of male and female cells obtained from a number of tissues and species. Several monoclonal antibodies appeared to be positive in all assays tested, suggesting that the molecule conferring the H-Y antigenicity lacks species-specificity and appears to be identical for soluble and membrane-bound H-Y antigen.

The most promising monoclonal antibodies reactive with the H-Y antigen have been evaluated for their efficiency in sexing Day 7 bovine preimplantation embryos (Chapter 4). Although in an indirect immunofluorescence assay a discrimination between male and female embryos could be made, evaluation of the staining patterns was fairly subjective because of non-specific binding of the monoclonal antibodies to the embryonic cells. After modifying the technique in order to reduce non-specific binding, the number of false positives after sexing under these conditions was greatly reduced, which suggests that the monoclonal antibodies detect a male-specific anti-

gen. It was concluded that the occurrence of false-negative embryos might be caused by a weak expression of the H-Y antigen and/or a low affinity of the monoclonal antibody for bovine cell-surface H-Y antigen. The antibodies used in our experiments had been selected on basis of their binding to both soluble and cell-surface H-Y antigen originating from different sources. Currently, monoclonal antibodies which are selected on high affinity for protein structural determinants on bovine cell-surface H-Y antigen are being produced in order to be used in a highly discriminating sensitive fluorescence assay.

In Chapter 1 has been discussed that it will take some time to fully realize the potential of monoclonal antibodies in the area of passive immunization or immunomodulation. The production costs are still relatively high and the reaction of the animal to the injected antibodies may limit the effects of passive immunization. Some such limitations are illustrated in the experiments in which anti-progesterone murine monoclonal antibodies were administered to cyclic pigs (Chapter 5). Intravenous injection of increasing amounts of anti-progesterone antibodies resulted in a concomitant rise in levels of antibody-bound progesterone. At the same time a significant rise in plasma concentrations of total progesterone was observed immediately after administration of higher doses of antibodies. Therefore, the net effect of progesterone binding by the antibody was relatively small and more or less independent of the quantities of antibody administered. It has been suggested that animals maintain adequate levels of free progesterone in their circulation by resorption of progesterone from a pool present in body tissues. The effects of administration of anti-progesterone antibodies on plasma levels of free progesterone are, however, not only influenced by the proposed compensating effect of resorption, but also by the possible initiation of a humoral response of the pigs to the injected antibodies. In the experiments described in Chapter 5 it is shown that a minimum dose of 32 mg anti-progesterone antibody elicited an anti-mouse response after the first injection, having an neutralizing effect on the anti-progesterone monoclonal antibodies administered with the second injection. When smaller quantities of antibody were used, an anti-mouse reaction was detected after the second or third injection.

From reports concerning the therapeutic use of monoclonal antibodies in man it is known that such an immune response may be directed partially against the isotypic determinants, partially against the idiotypic deter-

minants of the murine antibodies administered. Although anti-idiotypic responses cannot be excluded as a complicating factor, homologous antibodies might offer some advantages over their murine counterparts in terms of effectiveness for passive immunization.

In Chapter 6 the construction of a bovine-murine heteromyeloma cell line to be used for the production of bovine monoclonal antibodies has been described. It was anticipated that a heteromyeloma would retain the superior fusion characteristics of the mouse myeloma cells and, because of the presence of bovine chromosomes, would be better able to support stable bovine antibody production than interspecies hybridomas produced by fusing mouse myeloma cells with bovine lymphocytes. First (bovine-murine) and second generation (bovine-[bovine-murine]) fusion partners were compared for fusion efficiency and the generated number of antigen-specific antibody-producing clones. In addition, the optimal time-interval between boosting and harvesting of the lymphocytes for fusion and the source of lymphocytes was studied. It could be concluded that fusion of bovine lymph node cells with the second generation heteromyelomas on Day 7 after the final booster injection resulted in the largest number of specific antibody-producing clones. Experiments with a third generation bovine-murine heteromyeloma cell line indicated that fusion efficiency could be further improved (unpublished data). Studies with anti-rotavirus and anti-pregnant mare serum gonadotrophin (PMSG) bovine monoclonal antibodies, produced with the second generation fusion partners, indicate that the heteromyeloma cell lines are very useful for the production of bovine monoclonal antibodies.

The availability of such bovine monoclonal antibodies offers the possibility to compare the efficacy of homologous and heterologous antibodies in cattle after repeated passive immunization. The in vivo immunoneutralization of PMSG by murine and bovine monoclonal antibodies was chosen as a model for such a study (Chapter 7). Results indicate that repeated injection of murine monoclonal antibodies against PMSG (mMCA) alone did not, or only to a small degree, elicit an anti-mouse immune response. The simultaneous administration of mMCA and PMSG resulted in relatively high levels of anti-mouse antibodies after the second injection, leading to a decrease in neutralizing activity of mMCA. The results suggest that the neutralizing activity of mMCA is inhibited more by anti-idiotypic than by anti-isotypic antibodies against mMCA. After repeated administration of the bovine mono-

clonal antibody against PMSG (bMCA), either alone or in combination with PMSG, no anti-bMCA antibodies could be detected. In addition, no change in plasma levels of bMCA and PMSG, compared with levels after the first injection, was observed. Although it has to be confirmed by further experiments whether our findings can be generalized, the present results suggest that for repeated passive immunization in cattle homologous antibodies are to be preferred above heterologous antibodies. As far as we can see now it is necessary to evaluate carefully the need to produce homologous or heterologous antibodies, dependent on the amounts of antibody to be administered and the number of treatments.

In conclusion, the potential of monoclonal antibodies for diagnostic use, therapy or fundamental research, discussed in Chapter 1, together with the results presented in this thesis indicate that the monoclonal antibody technology will have an important impact on the improvement of animal quality and productivity.

POPULAIRE SAMENVATTING

Sinds Köhler en Milstein ongeveer vijftien jaar geleden de hybridomatechniek ontwikkelden, waardoor het mogelijk werd monoclonale antistoffen met de grootst mogelijke specificiteit in vrijwel onbeperkte hoeveelheden te produceren, heeft de toepassing van deze techniek in het medisch-biologisch onderzoek een stormachtige ontwikkeling doorgemaakt. Monoclonale antistoffen zijn thans ook niet meer weg te denken uit het onderzoek in de dierlijke produktie.

Belangrijke toepassingsgebieden van monoclonale antistoffen in de dierlijke produktie zijn de diagnostiek, de beïnvloeding van fysiologische processen door middel van passieve immunisatie en het fundamentele onderzoek. In Hoofdstuk 1 van dit proefschrift worden enkele toepassingen van monoclonale antistoffen op genoemde gebieden, met name op het terrein van de voortplanting, besproken. De voor- en nadelen van monoclonale antistoffen ten opzichte van polyclonale antisera, alsmede de beperkingen van de huidige techniek en de te voorziene verbeteringen dienaangaande in de komende jaren, worden uitvoerig beschreven.

Als voorbeeld van het gebruik van monoclonale antistoffen in de diagnostiek wordt in Hoofdstuk 2 de produktie en karakterisering van monoclonale antistoffen tegen progesteron beschreven voor toepassing in de melkprogesterontest. Via bepaling van het progesterongehalte in de melk kan worden vastgesteld of een koe al dan niet tochtig of drachtig is. Hoewel de verkregen monoclonale antistoffen, wat specificiteit en affiniteit (= de mate van binding) voor progesteron betreft, niet beter waren dan een reeds beschikbaar polyclonaal antiserum, werd toch uit het oogpunt van standaardisatie op basis van een monoclonale antistof een "cow-side test" ontwikkeld.

Een tweede voorbeeld van toepassing van monoclonale antistoffen in de diagnostiek betreft het bepalen van het geslacht van runderembryo's voorafgaand aan transplantatie, met behulp van antistoffen tegen het H-Y antigeen. Het H-Y antigeen is een anti-transplantatie antigeen dat op het oppervlakte-membraan van uitsluitend mannelijke cellen voorkomt. In Hoofdstuk 3 is de produktie en karakterisering van monoclonale antistoffen tegen het H-Y antigeen beschreven. Enkele antistoffen werden verkregen die zowel een goede binding gaven met oplosbaar H-Y antigeen afkomstig van meerdere bronnen alsook met membraan-gebonden H-Y antigeen van verschillende typen cellen afkomstig van diverse diersoorten.

De daarvoor het meest in aanmerking komende monoclonale antistoffen tegen het H-Y antigeen zijn vervolgens toegepast in een indirecte fluorescentietest voor het onderscheiden van het geslacht van runderembryo's (Hoofdstuk 4). De nauwkeurigheid van deze test bedroeg aanvankelijk 77%, waarbij zowel vals-positieve als vals-negatieve uitslagen voorkwamen. Na optimalisatie van de testcondities was de nauwkeurigheid lager, namelijk 73%. Er kwamen echter geen vals-positieve uitslagen meer voor, wat betekent dat de antistoffen specifiek mannelijk zijn.

In Hoofdstuk 5 is beschreven in hoeverre monoclonale antistoffen tegen progesteron gebruikt kunnen worden voor de beïnvloeding van fysiologische processen door middel van passieve immunisatie. Het bleek dat na toediening van verschillende hoeveelheden antistoffen aan cyclische gelten de plasma progesteron spiegels (d.w.z. het niet aan de antistoffen gebonden progesteron) slechts in zeer geringe mate en voor korte tijd gereduceerd waren. Zodra het progesteron in de bloedbaan aan de antistoffen wordt gebonden, komt mogelijk progesteron vrij dat in het vetweefsel in grote hoeveelheden is opgeslagen. De voor de weefsels beschikbare hoeveelheid progesteron blijft daardoor min of meer constant. Bij de experimenten kwam eveneens naar voren dat bij het varken na herhaald toedienen een immuunrespons wordt opgewekt tegen de monoclonale antistoffen. De monoclonale antistoffen zijn van muize-origine en het varken herkent deze als lichaamsvreemd.

De anti-muis reaktie bij het varken was mede aanleiding om een systeem te construeren voor de produktie van voor landbouwhuisdieren homologe antistoffen (Hoofdstuk 6). Door de muize myeloma cellen enkele keren te fuseren met lymfkliercellen van het rund, werd uiteindelijk een rund-muis heteromyeloma cellijn verkregen waarmee op relatief efficiënte wijze runder monoclonale antistoffen kunnen worden geproduceerd. In dit kader werd ook aandacht besteed aan de geschiktheid van antigeen-gestimuleerde runder miltcellen ten opzichte van lymfkliercellen voor fusie met de heteromyeloma cellijn, alsmede aan het optimale tijdstip voor fusie na de laatste antigeen-stimulatie. Tijdens het gehele proces van ontwikkeling van de heteromyeloma cellijn zijn runder monoclonale antistoffen geproduceerd tegen rotavirus en pregnant mare serum gonadotrophine (PMSG).

Doordat ook muize monoclonale antistoffen tegen PMSG commercieel verkrijgbaar zijn, kon tenslotte een vergelijkende studie verricht worden naar de geschiktheid van muize monoclonale antistoffen ten opzichte van runder monoclonale antistoffen voor neutralisatie van PMSG in vivo na herhaalde toediening bij het rund (Hoofdstuk 7). Met name bij gelijktijdige

toediening van de muize monoclonale antistoffen en PMSG werden direkt na de 2e injectie en in nog sterkere mate na de 3e injectie anti-muis antistoffen aangetoond. Tegelijkertijd nam de neutralisatie van PMSG door de muize monoclonale antistoffen af. Na herhaalde toediening van runder monoclonale antistoffen, al dan niet in combinatie met PMSG, konden geen antistoffen gericht tegen de runder monclonalen worden aangetoond. Voorlopige resultaten wijzen er dan ook op dat bij herhaald toedienen van monoclonale antistoffen de voorkeur gegeven dient te worden aan homologe in plaats van heterologe antistoffen.

Door het uitwerken van enkele typische voorbeelden van toepassingen van monoclonale antistoffen in de diagnostiek en door het onderzoek naar het effectief toepassen van monoclonale antistoffen in het kader van de beïnvloeding van fysiologische processen door middel van passieve immunisatie, alsmede door het bespreken van enkele voorbeelden van toepassingen in het fundamentele onderzoek, kan dit proefschrift bijdragen tot het verkrijgen van meer inzicht in de waarde van monoclonale antistoffen voor de dierlijke produktie.

CURRICULUM VITAE

Peter Booman werd op 22 september 1955 geboren te Krimpen aan den IJssel. In 1973 behaalde hij het Gymnasium-β diploma aan de Gereformeerde Scholengemeenschap te Rotterdam. In juni 1980 studeerde hij cum laude af aan de Landbouwuniversiteit te Wageningen in de studierichting Zoötechniek met als afstudeervakken de Gezondheids- en Ziekteleer der Huisdieren, de Dierfysiologie en de Industriële Bedrijfskunde. Na zijn afstuderen werd hij aangesteld als wetenschappelijk medewerker in tijdelijke dienst bij het Instituut voor Veeteeltkundig Onderzoek "Schoonoord" te Zeist. Na aanvankelijk betrokken te zijn geweest bij het endocrinologisch voortplantingsonderzoek, werd hij vanaf september 1981 verantwoordelijk voor het immunobiologisch onderzoek. In maart 1985 is de tijdelijke aanstelling omgezet in een aanstelling in vaste dienst. Vanaf juli 1988 is hij, naast hoofd van de afdeling Immunobiologie, tevens teamleider van de werkgroep Voortplanting.