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A Bovine Monoclonal Antibody to Oestrone/Oestradiol Prepared by a (Murine×Bovine) ×Bovine Interspecies Fusion

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(Accepted 4 June 1987)

ABSTRACT

Groves, D.J., Clayton, J. and Morris, B.A., 1988. A bovine monoclonal antibody to oestrone/oestradiol prepared by a (murine×bovine) ×bovine interspecies fusion. *Vet. Immunol. Immunopathol.*, 18: 95-101.

A heterohybridoma was produced by the fusion of sensitized peripheral blood lymphocytes (PBLs) with a previously derived heteromyeloma, generated by the fusion of bovine PBLs with murine myeloma cells. The sensitized bovine PBLs were collected from a steer immunized with an oestradiol-ovalbumin conjugate. The cell lines resulting from the fusion were screened for the production of bovine antibodies to oestradiol. A stable heterohybridoma was isolated which secreted a bovine IgG₁ to oestrone/oestradiol.

The use of sensitized PBLs together with heteromyeloma fusion partners has proved to be a reliable and simple way of producing monoclonal antibodies against specific haptens.

INTRODUCTION

Recent investigations into the neutralization of steroids in domestic livestock by passive immunization have demonstrated the usefulness of the technique as a way of increasing fecundity in sheep and, to a lesser extent, in cattle (Webb et al., 1984). Passive immunoneutralization necessitates the production of large amounts of allogeneic antisera, especially for work with cattle. The inherent variability of these polyclonal antisera, combined with the relatively poor immunological response of cattle, has made the development of monoclonal bovine antibodies an important aspect of this research.

Since there is no bovine myeloma line readily available for sensitization to selective agents, the immortalization of peripheral blood lymphocytes from immunized steers was attempted by fusion with murine myeloma cells.

Interspecific hybridization often results in rapid, extensive and preferential

loss of chromosomes of the non-myeloma species (McDermott, 1975). However, in the murine \times human cross, there is evidence that the loss of human chromosomes is not random and that some are preferentially retained (Kozbor and Croce, 1985). Teng et al. (1983) and Tucker et al. (1984) replaced the usual mouse myeloma line with a heteromyeloma, produced by the fusion of human or bovine lymphocytes respectively with a mouse myeloma line. This strategy reduced the loss of non-murine chromosomes and the associated disruption of gene expression in the resulting hybridomas. The current paper reports the use of an interspecific fusion partner, generated by sensitizing a murine \times bovine hybridoma line to selective media. This heteromyeloma was then used to produce a monoclonal cell line secreting a bovine antibody to oestrone/oestradiol.

MATERIALS AND METHODS

Harvesting of lymphocytes

A Friesian steer was immunized and boosted with oestradiol-6CMO-ovalbumin in Freund's adjuvant. Five days after boosting, 200 ml of blood were taken from the jugular vein into lithium heparinized vacutainers (Becton Dickinson). Blood was diluted 1:1 with sterile phosphate-buffered saline, pH 7.4 (Flow Labs.). The lymphocytes were separated by density gradient centrifugation on lymphopaque (Nyegaard, U.K.), followed by two washes in Hank's balanced salts solution (Gibco). The lymphocytes were then resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% foetal calf serum (FCS) (Gibco).

Fusion

The preparation of the (bovine \times murine) heteromyeloma, B/MF-2, has been described previously (Groves et al., 1987a, b). Exponentially dividing B/MF-2 cells (0.5×10^7) and peripheral blood lymphocytes (4×10^7) were fused using 50% w/v polyethylene glycol 1500 (BDH) added over a 2-min period, followed by gradual dilution with 10 ml of L-15 Leibovitz medium (Gibco) over 6 min. Cells were washed with L-15, resuspended in 24 ml of DMEM/10% FCS containing hypoxanthine, aminopterin and thymidine (HAT). One-ml aliquots were then dispensed into each well of a Nunc 24-well multidish over a monolayer of mouse thymocytes as feeder cells. Cultures were incubated at 37°C in an atmosphere of 5% CO₂, 95% air in a non-humidified automatic CO₂ incubator (Model; GA3, LEEC). After 7 days, 50% of the culture medium was aspirated and replaced with DMEM/10% FCS containing HT only. This medium was subsequently replaced with DMEM/10% FCS.

Screening assay

Cell supernatants were screened for bovine antibodies to oestradiol using an ELISA. The assay system was similar to that described by Groves et al. (1987b) for the assay of bovine anti-testosterone antibodies, except that a β -oestradiol-6CMO-bovine serum albumin conjugate (Sigma) was used as the coating antigen at a concentration of $1\mu\text{g/ml}$. An $(\text{NH}_4)_2\text{SO}_4$ -precipitated polyclonal IgG preparation of a bovine anti-oestradiol serum was used as the standard.

The screening assay was designed as a rapid and reproducible system to specifically detect bovine antibodies reacting with the coating steroid-conjugate.

Internal standards were incubated on each assay plate to correct measured concentrations for inter-assay variability. The coefficients of variation for bovine polyclonal antiserum, diluted 1:100 000 in phosphate buffer, was 8.09% (intra-assay) ($n=12$) and 9.85% (inter-assay) ($n=60$). The detection limit of the assay was 0.839 ng/ml of specific antibody (equivalent to a fall in binding of $2\times\text{SD}$ from the zero wells). Mouse IgG ($20\mu\text{g/ml}$) did not interfere with the assay.

Antibody characterization

Antibody cross reactivities determined in solid phase assays may not accurately reflect the aqueous in vivo situation due to alterations in the conformation of the immobilized immunoreagent on assay plates (Vaidya et al., 1985). As these antibodies were being developed for in vivo use, the specificity of the monoclonal antibody was therefore determined in a liquid phase radioimmunoassay (RIA), using a tritiated tracer. The equilibrium dissociation constant (K_D) and the concentration of antibody binding sites were calculated, using the same RIA system, from equilibrium binding studies performed at 4°C (Scatchard, 1949; Rosenthal, 1967). Culture medium containing anti-oestrogen antibodies was incubated overnight in 0.04 M phosphate buffer (pH 7.4) with a fixed amount of tritiated oestradiol, 0.038 pmol/tube (or oestrone 0.057 pmol/tube) in the presence of increasing amounts of unlabelled oestradiol (0.039–1.000 pmol/tube) or oestrone (0.039–100.000 pmol/tube). Bound and free steroids were separated using a dextran-coated charcoal suspension in phosphate buffer, and the amount of antibody-bound radioactivity was measured by liquid scintillation counting. Data from these studies were analysed by the methods of Scatchard (1949) and Muller (1977).

The antibody class was determined by an ELISA. Plates were coated with oestradiol-6CMO-BSA; the samples were then incubated in the wells for 2 h at 37°C . The plates were washed and class-specific rabbit antibodies to bovine IgG₁, IgG₂, and IgM (Miles) were incubated in the plates for 2 h at 37°C . The plates were then washed before adding peroxidase-labelled donkey anti-rabbit

TABLE 1

Antibody characterization

Cell line	B/ME.8.10.B6
Antibody class	IgG ₁
Dissociation constant (K_D)	5.33×10^{-10} M
Secretion rate in DMEM/10% FCS	20 μ g specific antibody/ 10^6 cells per 24 h
Cross-reacting steroids	% Cross reactivity
Oestrone (1.3.5(10) estratrien-3-ol-17-one)	100%
Oestradiol (1.3.5(10) estratrien-3,17 β -diol)	33.5%
Dihydrotestosterone (5-androsten-3,17-dione)	2.4%
Progesterone (4-pregnan-3,20-dione)	0.9%
Androstenedione (4-androsten-3,17-dione)	0.7%
Testosterone (4-androsten-17 β -ol-3-one)	0.3%

IgG (Guildhay Antisera, University of Surrey) for 2 h at 37°C and visualized with orthophenylenediamine.

The specificity of the assay was checked by displacement of the anti-class antibodies by bovine IgG₁ and IgG₂ (Jackson IR, PA, U.S.A.) and bovine IgM (Calbiochem).

RESULTS

Cell culture

Following the fusion of B/MF-2 heteromyeloma cells with sensitized lymphocytes, hybridoma colonies were first recorded 8 days post-fusion in 13% of the wells. Positive colonies were first detected by ELISA at 9 days post-fusion. After 17 days, 33.3% of wells contained colonies and 37.5% of these colonies produced positive supernatants. At 13 days post-fusion, the four colonies secreting the highest levels of antibody were cloned by limiting dilution in Nunclon microwell plates over mouse thymocyte feeder layers. Of the resulting clones, 34.3% were positive for bovine anti-oestradiol. Positive colonies were expanded and recloned 38 days post-fusion. Of the resulting colonies, 81.0% produced bovine anti-oestradiol. Hybridoma B/ME.8.10.B6 was expanded for characterization. This clone was maintained in static batch culture in DMEM with 5% or 10% FCS or in Gibco serum-free hybridoma medium (high and low protein formulations) for up to 6 months before being frozen in liquid nitrogen.

Antibody characterization

Initial examination of antibody cross reactivity with a panel of different steroids revealed a very high degree (300%) of cross reactivity of the puta-

tive anti-oestradiol antibody with oestrone. Cross reactivities are therefore given in terms of oestrone (100%) rather than oestradiol (Table 1).

DISCUSSION

There have been many studies on the use of endogenous and exogenous antibodies to gonadal steroids as a means of modulating mammalian reproductive function (e.g. Webb et al., 1984; Rhind et al., 1985). Active immunization against androstenedione has progressed to the stage of commercial exploitation. The use of passive immunomodulation of ovarian steroid feedback on the pituitary, to increase the fecundity of domestic animals, has been demonstrated with polyclonal antisera (Webb et al., 1984).

The heterogeneity of polyclonal antisera, in terms of affinity, specificity and titre, makes it desirable to have a monoclonal preparation to provide a source of uniform antibody.

Four separate groups of workers have reported the production of monoclonal bovine immunoglobulins, all using the fusion of murine myeloma cells with bovine lymphocytes. Srikumaran and co-workers have produced monoclonal bovine immunoglobulins of different classes, from fusions of SP2/0 murine myeloma cells with either normal bovine lymphocytes or with lymphocytes from animals immunized against DNP conjugates (Guidry et al., 1986) and used as sources of uniform preparations of bovine immunoglobulin classes. Tucker and her colleagues have developed a number of bovine monoclonal antibodies against blood group determinants (Tucker et al., 1984, 1987; Anderson et al., 1986) and bacterial antigens (Anderson et al., 1987) using heteromyelomas as well as NSO mouse myeloma cells as the immortal fusion partners. Davidson et al. (1982) reported the production of a bovine IgM of undefined specificity by fusing bovine leukemic lymphocytes with NS1 or X63 mouse myeloma cells. Raybould et al. (1985) have produced a bovine IgG₂ reacting with bovine enteric coronavirus by fusing sensitized lymphocytes with SP2/0 cells. Our own laboratory has recently reported the isolation of a (murine × bovine) × bovine hybridoma secreting a bovine IgG₁ directed against testosterone (Groves et al., 1987b).

The heteromyeloma used in the current study was generated in our laboratory, by the fusion of NS1 mouse myeloma cells and bovine lymphocytes, and its use was first reported by Groves et al. (1987b). The fusion ratio of lymphocytes:heteromyeloma cells in this study was 8:1 with a total cell number of 4.5×10^7 for each fusion. This compares with the conditions used by Srikumaran et al. (1984) (1:1; 2×10^8), Groves et al. (1987b) (4:1; 5×10^7), and Tucker et al. (1984) (10:1; 2.5×10^8).

Secretion levels obtained were of a similar order to those reported in other interspecies fusions (e.g. Nowinski et al., 1983; Teng et al., 1983; Abrams et al., 1984; Groves et al., 1987b). Comparison of production rates of different

bovine × murine hybridomas is difficult because of the lack of uniformity of expression of this parameter. The stability of the cell line B/ME.8.10.B6 was established after repeated cloning and the antibody secretion was not affected by repeated passaging of the cells or by freezing in liquid nitrogen and subsequent thawing.

Further studies on the optimization of production and purification processes for bovine monoclonal antibodies are underway. We hope that it will soon be possible to conduct field trials to assess the in-vivo activity of these antibodies. Antisera to oestrone and to oestradiol have been shown to exert similar effects on FSH and LH levels and patterns of secretion (Pathiraja et al., 1984). Both antisera are effective in increasing ovulation rate and lambing rate in Welsh mountain ewes. Since the most effective use of passive immunization has been shown to be the administration of a mixture of antisera to different steroids (Land et al., 1982) the specificity of the reported monoclonal antibody to oestrone/oestradiol should not impair its usefulness.

Many previous studies have encountered difficulty in the establishment of stable interspecific hybridomas and this problem has been overcome, at least in part, by the use of a murine × bovine heteromyeloma fusion partner. The simplicity of the system has also been improved by using easily obtained peripheral blood lymphocytes in place of the more usual, terminally obtained, spleen or lymph node B-cells.

The routine production of monoclonal antibodies of pre-defined specificity from domestic animals is sure to broaden the veterinary applications of hybridoma technology.

ACKNOWLEDGEMENTS

We would like to express our gratitude to Professors D.V. Parke, V. Marks and R. Spier in whose departments this work was carried out. The work was supported by grant 90/9 from the A.F.R.C. to B.A.M.

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