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Production of Monoclonal Antibodies specific for Progesterone

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Abstract: Progesterone levels in milk and serum are indicators of pregnancy in cattle. The progesterone level reaches a peak on the 21st and 22nd days of pregnancy. Monoclonal antibodies specific to progesterone could be used for the immunodetection of milk and serum progesterone levels. We report here the development of hybrid cells prdoducing monoclonal antibodies specific for progesterone using hybridoma technology. Hybridoma cells secreting monoclonal antibodies against progesterone (MAM 2H11, MAM 6G6) were developed by fusion of the spleen cells of an immunized BALB/c mouse with FO (ATCC CRL 1646) mouse myeloma cells. These monoclonal antibodies were characterized for use in the development of diagnostic kits based on the enzyme-linked immunosorbent assay (ELISA) test system. ELISA tests showed that antibodies produced by 2 hybrids from 375 wells reacted specifically with progesterone. Monoclonal antibodies did not show any cross reaction with other steroid hormones.

Key Words: Immunodiagnosis, monoclonal antibody, progesterone, ELISA

Progesteron'a Özgü Monoklonal Antikorların Üretilmesi

Özet: Progesteron hormonunun süt ve serumdaki miktarı gebeliğin bir göstergesidir. İneklerde gebeliğin 21. ve 22. günlerinde progesteron miktarı yüksektir. Progesterona karşı geliştirilmiş monoklonal antikorlar ile süt ve serumdaki progesteron düzeyi belirlenebilmektedir. Bu çalışmada hibridoma teknolojisi kullanılarak progesterona özgün monoklonal antikor üreten hibrit hücrelerinin elde edilmesi amaçlanmıştır. Progesterona karşı monoklonal antikorlar sentezleyen hibridoma klonları (MAM 2H11, MAM 6G6), FO (ATCC CRL 1646) fare myeloma hücrelerinin, immünize edilmiş (progesteron ile) BALB/c farelerinin dalak hücrelerinin birleştirilmesi sonucu geliştirilmiştir. Bu monoklonal antikorlar enzim işaretli immünolojik deney (ELISA) sistemine dayalı tanı kitlerinin geliştirilmesinde kullanılmak üzere karakterize edilmiştir. Füzyonlar sonucunda 375 kuyuda hibrit klon gözlenmiş bu klonlar içerisinde 2 tanesinin ürettiği antikorların progesteron ile özgün reaksiyon verdiği (ELISA) belirlenmiştir. Elde edilen monoklonal antikorlar diğer steroid hormonlar ile çapraz tepkime göstermemiştir.

Anahtar Sözcükler: İmmün teşhis, monoklonal antikor, progesteron, ELISA

Introduction

Progesterone is a steroid hormone with a molecular weight of 314 daltons and is secreted during pregnancy. Early diagnosis of pregnancy is very important in increasing the fertility rate of cattle. In cattle, the progesterone level reaches a peak on the 21st and 22nd day of pregnancy (1, 2). Veterinary scientists routinely use the presence of progesterone hormone in milk and serum in pregnancy testing in dairy cattle (3, 4).

Fantl et al. produced a monoclonal antibody against this antigen (5). In addition, two high affinity antibodies were also produced in a single fusion experiment in which nine monoclonal antibodies were developed against progesterone (17).

Hybridoma cells were generated by the fusion of a myeloma cell line with antibody-secreting lymphocytes from an immunized mouse and an antibody of unique specificity was obtained by isolating individual hybridoma clones.

This report describes the development of a hybridoma line producing monoclonal antibodies with high specificity for progesterone with the ultimate aim of developing an immune diagnostic kit prototype using these monoclonal antibodies.

Material and Methods

Reagents

Progesterone 3(O- Carboxymethyl) oxime conjugated to bovine serum albumin (BSA.P) (steroids: BSA, 38:1) was obtained from the SIGMA company. All the other steroids; the antigens β-estradiol 6-(O- Carboxymethyl) oxime conjugated to bovine serum albumin (BSA.E); testosterone conjugated to bovine serum albumin (BSA.T); aldosterona conjugated to bovine serum albumin (BSA.A); corticosterone conjugated to bovine serum albumin (BSA.C); and Dexamethazone, were also obtained from SIGMA.

Dulbecco's Modified Eagles Medium (DMEM), antibiotics, Hypoxanthine, aminopterine, thymidine (HAT) and hypoxanthine thymidine (HT) were purchased from GIBCO.

Immunization

Progesterone dissolved in phosphate buffered saline (PBS: 10 mM K_2HPO_4, 10mM KH_2PO_4, 0.15 mol/ L NaCl, pH 7.2) and mixed with equal volumes of Freund's complete adjuvant and 0.2 ml (30 µg) of progesterone was given by intraperitonal injection to five eight-week-old BALB/c mice. Weekly booster injections were given for 3 weeks according to the method described above using incomplete instead of complete Freund's adjuvant. Four days before removal of the spleen for cell fusion, intravenous injection of 20 µg of progesterone in 0.1 ml PBS without adjuvant was administered.

Selection of Spleen Donors

The antibodies were measured in tail-vein blood samples from the immunized mice. The blood was collected in a microfuge tube containing sodium citrate. The tubes were centrifuged

at 6000 rpm./ min to remove the blood cells. The plasma samples were tested using the ELISA test system. Plasma from a non-immunized mouse in PBS was used as the control. A mouse found to be responsive to immunization against progesterone was selected as the spleen donor for fusion.

Fusion

3.10^7-5.10^7 myeloma cell line F0 (ATCC CRL 1646) and 3.10^8 spleen cells (immunized with progesterone) were fused in ratios between 1:6 and 1:10 using polyethylene glycol 4000 as fusing agents (6, 7, 8). After fusion, the cells were distributed into the wells of 96 culture plates (NUNC) at a cell density of 30000 cells per well and cultivated in the standard medium (DMEM) supplemented with 20% fetal calf serum (FCS, Biochrom), 100 mM hypoxanthine, 0.4 µM aminopterine and 16 µM thymidine (HAT). After 14-21 days, hybrid cells showing antibody activity against progesterone, estradiol and testosterone were cultured again using a layer of feeder cells (macrophages) from a non-immunized BALB/c mouse to assist in the early stages of growth. Positive hybrid clones producing antibody with the highest specificity were subcloned by limiting dilution (9). At each stage of growth, aliquots of the hybrid cultures (3-5.10^6 cells) were frozen in liquid nitrogen in 80% DMEM, 20% FCS and 10% dimethylsulphoxide.

ELISA

The indirect enzyme-linked immunosorbent assay (ELISA) (10, 11) was used for the screening of the hybridoma supernatant for anti-progesterone activity. 96 well polystrene plates (NUNC immunoplates) were coated with 200 ng BSA.P in parallel with BA.E, BSA.T., BSA.C., BSA.A., Dexamethazone and BSA, for cross reaction in 100 µl PBS. The coating of the plates was carried out at 4˚C overnight. The plates were washed three times with washing buffer (0.005% tween-20 in PBS). Then, 0.2% milk powder in PBS was added to the wells and the plates were incubated for 1 h at 37˚C followed by washing as above. Each hybridoma supernatant was tested with each steroid (BSA.P., BSA.E., BSA.T., BSA.C., BSA.A. and Dexamethazone) and the plates were incubated at 37˚C for 1 h. After washing, alkaline phosphatase conjugate of polyvalent goat-antimouse Ig (SIGMA) in 1:1000 dilution buffer (PBS) was added to each well and incubated for 1 h at 37˚C. After washing five times with washing buffer, as described above, the substrate buffer (1 mM $ZnCl_2$, 1mM $MgCl_2$, 0.1 M glycine pH 10.4) and para-nitrophenyl phoshate mg/ml at 405 nm were determined.

Purification of Monoclonal Antibodies and Subisotyping

Monoclonal antibodies were purified from the hybridoma supernatant by ammonium sulfate $(NH_4)_2SO_4$ precipitation at between 30 and 50% saturation. For precipitation of monoclonal antibodies from the culture supernatant, crystalline ammonium sulfate (341 g/L) was slowly added with stirring and left overnight at 4˚C to ensure precipitation of all the proteins. The mixture was centrifuged for an hour at 13000 rpm at 4˚C, and the pellet was resuspended in 10 ml PBS and dialyzed against 100 times volume of PBS for 24 hours at 4˚C. This was later subjected to immunoaffinity chromatography using solid-phase bound protein G (S.aureus) as the IgG-immunotrap (Mab-Trap/Pharmacia) (12). Ig typing of the monoclonal antibodies was performed using a hybridoma subisotyping kit (Boehringer Mannheim).

Affinity Measurements

Affinity measurements of the monoclonal antibodies were carried out by equilibrium dialysis, using radiolabelled (^{3}H) progesterone (NEN, specific activity; 109.5 Ci/mmol). Antigens and antibodies were incubated in equilibrium dialysis cells for 20 h at room temperature and shaken slowly. Radioactivity measurements were done in an LKB (Wallac) 1212 Rackbeta Liquid Scintillation Counter. The resulting slope was estimated using a graFit computer program and dissociation constants were determined according to the method of Scatchard (13).

Results

Cell Fusion and Antibody Production

The results of the fusion following immunization with progesterone antigen are summarized in Table 1. Two hybridoma cell lines (MAM 2H11, MAM 6G6) producing specific monoclonal antibodies to progesterone were selected from this fusion.

Table 1. Results of Fusion

	Fusion 1	Fusion 2
Number of spleen cells	3.10^8	3.10^8
Number of myeloma cells (FO ATCC CRL 1646)	5.10^7	3.10^7
Total number of wells used for the fusion	672	672
Number of hybrid cell clones	135	240
Number of hybrid cell clones having antibody activity	5	11
Number of hybrid cells producing specific antibody for progesterone	1	1

Table 2 shows the clones producing antibodies with high specificity for BSA. progesterone. For this assay, the supernatant medium was tested for antibody activity for progesterone and these antibodies were also tested for cross reaction with the other steroid hormones. Only two of them, MAM 2H11 and MAM 6G6, did not show any cross reaction with estradiol, testosterone, aldosterone, corticosterone and dexamethosone.

Steroids used in ELISA	*Hybrids*	
	MAM-2H11	MAM-6G6
Progesterone	1.35	1.37
Estradiol	0.24	0.28
Testosterone	0.23	0.23
Aldosterone	0.22	0.24
Corticosterone	0.22	0.24
Dexamethazone	0.23	0.25
BSA	0.25	0.26
No antigen (blank)	0.11	0.11

Table 2. Comparison of the reactivity (OD405) of the monoclonal antibodies (MAM 2H11, MAM6G6) with different steroids.

Characterization of Antibodies

From the analysis of the supernatant samples, two progesterone-specific monoclonal antibodies were obtained. Monoclonal antibodies were identified as the IgG2a substype using a hybridoma subisotyping kit system. They also contained kappa light chains. The affinity of these monoclonal antibodies for progesterone was determined by equilibrium dialysis. The affinity constants (K_d, M) measured for the purified monoclonal antibodies are shown in Table 3.

Monoclonal Antibody	K_dM	Immunoglobulin class
MAM 2H11	$2.1x10^{-8}$	IgG2a
MAM 6G6	$1.2x10^{-9}$	IgG2a

Table 3. Apparent dissociation contants (Kd) and immunoglobulin class of monoclonal antibodies obtained using immunization with BSA.P

Discussion

Heap demonstrated that concentrations of progesterone in milk and serum are one of the determining factors for the establishment of pregnancy in dairy cattle after insemination (14). As a result, monoclonal antibodies against progesterone are used in early pregnancy testing in dairy cattle. In this study, we used the method of Köhler and Milstein (15) to generate hybrid cell lines producing antibodies to progesterone.

Using hybridoma technology, we developed hybrid cells producing antibodies for progesterone (MAM 2H11, MAM 6G6). The affinities of these antibodies were found to be 10^{-8}-10^{-9} M. Some groups also developed monoclonal antibodies against 11 α-hydroxyprogesterone hemisuccinate and 6β-hydroxyprogesterone hemisuccinate. These results are in agreement with other monoclonal antibody studies in the literature (5, 16, 17, 18).

The results of fusion are summarized in Table 1. Sixteen progesterone-reactive clones were obtained in two fusions, two of which proved to be progesterone specific. They exhibited moderate to negligible reactivity with the other steriods when tested with ELISA (Table 2).

The results show that the monoclonal antibodies (MAM 2H11, MAM 6G6) can be used efficiently in the early and sensitive digagnosis of progesterone by immune diagnostic test systems (i.e. ELISA).

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