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Production and Specificity of Monoclonal Antibodies Reactive to Progesterone

Chung-Boo KANG[*1], Ikuo DOMEKI[*2], Hideo KAMOMAE[*3], Toshio IKEDA[*3], Masayoshi YUKAWA[*4] and Takashi ONODERA[*5]

[*1,3,5] Third Research Division, National Institute of Animal Health (Tsukuba, Ibaraki, 305 Japan)
[*2] Animal Reproduction Division, National Institute of Animal Industry (Tsukuba, Ibaraki, 305 Japan)
[*4] Department of Biomedical Science, Faculty of Agriculture and Veterinary Science, Nihou University (Fujisawa, Kanagawa, 252 Japan)

Abstract
Monoclonal antibodies to progesterone 11α-hydroxy hemisuccinate were prepared. Supernatant of clone hybridoma reacted positively to the progesterone solution. Dot-blot analysis revealed a single precipitation band when rabbit anti-mouse IgM and anti-mouse κ were used. These monoclonal antibodies did not react with pregnenolone, testosterone, estrone, estradiol-17β, aldosterone, hydrocortisone (cortisol), cortisone, corticosterone and 11α-dehydroxycortisone (DOC). Radioimmunoassay using those monoclonal antibodies was developed. Data from this assay supported the following conclusions: (1) association constant (K_A) of supernatant of clone PrM for progesterone was 2×10^{10}, (2) monoclonal antibodies reacted to progesterone most efficiently at 1 : 10,000 dilution, and (3) efficiency of reactivity of monoclonal antibodies was higher than that of rabbit antibodies.

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Radioimmunoassay (RIA) has been the major analytical technique used in endocrinological studies during the last 20 years. Many steroid enzyme-linked immunosorbent assays (ELISA) currently available employ microtiter plates for the determination of progesterone. In RIA and ELISA, monoclonal antibodies are more specific to the steroid hormones than polyclonal antibodies. Our present report describes a sensitive, rapid, solid-phase microtiter plate assay using monoclonal antibodies for the detection of progesterone. This assay, validated by comparing the results obtained by ELISA[4] with those of reliable RIA[1,2], has been used routinely in our laboratory over the past 6 months, and could be applied extensively in the general field of hormone assay.

The antiserum against progesterone 11α-

Present address: [*1] Department of Veterinary Medicine, Gyeongsang National University (Jinju, Gyeongam, Korea)
[*5] Faculty of Agriculture, the University of Tokyo (Yayoi, Bunkyo, Tokyo, 113 Japan)
Correspondence should be addressed to T. Onodera.

hydroxy-hemisuccinate-BSA (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was produced in male Japanese white rabbits, 3- to 5-months old, by the immunization protocol described previously[6]. Each rabbit received 1 m*l* (1 mg/m*l*) of the emulsified immunogen at 0, 2 and 4 weeks with a booster (1 mg) administered every 3 weeks thereafter until the titer did not increase further. Rabbits produced highly specific antisera against progesterone 11α-hydroxy-hemisuccinate-BSA by day 90, and 1:2,000 diluted sera were used for RIA and ELISA.

Monoclonal antibodies against progesterone 11α-hydroxy-hemisuccinate were produced using BALB/c mice as described previously[3]. Antigen was dissolved in phosphate-buffered saline (PBS), emulsified with one third volume of Freund's complete adjuvant. Six to eight weeks old female BALB/c mice were injected intraperitoneally with 0.3 m*l* (containing 1 mg/m*l* antigen) of emulsion. At intervals of 2 weeks, booster injections were given as indicated above except that incomplete Freund's adjuvant was used. Three days before the removal of the spleen for cell fusion, an intravenous injection of 10 μg of antigen in 0.1 m*l* PBS was given.

Cell-fusion was performed between hyperimmunized mouse splenic lymphocytes (1×10^8) and mouse myeloma cells (1×10^7, P3U1) using 1 m*l* 50% polyethylene glycol 4,000 (Merck, Rahway, New Jersey, U.S.A.). Hybrid cells were selected by using a hypoxanthine, aminopterin and thymidine (HAT) medium. Two weeks later hybridomas of these wells were cultured in serum-free media (ASF 104, Ajinomoto, Tokyo, Japan). Using these supernatants, an indirect ELISA for screening for progesterone was performed as described previously[4]. Clone PrM, which gave a high optimal density for the screening process was selected for proliferation.

Clone PrM was cultured in ASF 104 at a concentration of 1×10^6 cells/m*l* for 4 days.

Table 1. Specificity of monoclonal antibody raised against progesterone 11α-hydroxy-hemisuccinate conjugated to bovine serum albumin

Steroid	Cross-reaction (%)
Progesterone	100
Pregnenolone	0.02
Testosterone	0.116
Estrone	<0.001
Estradiol-17β	<0.001
Aldosterone	0.008
Hydrocortisone (cortisol)	0.007
Cortisone	0.009
Corticosterone	0.997
DOC (11α-dehydroxycortisone)	2.179

The supernatant was subjected to ultrafiltration to obtain a 10-fold concentrated supernatant. Immunoglobulin class was determined by the dot-blot analysis system (Bethyl Laboratories, Montgomery, Texas, U.S.A.). A single precipitation band appeared in the supernatant of clone PrM when using rabbit anti-mouse IgM and anti-mouse κ. No precipitation band was formed with anti-mouse IgG_1, IgG_2, IgG_3 and λ. Specificity and association constant of the supernatant of clone PrM were determined according to the method of Abraham[1] and Steward[5], respectively on the cell culture supernatants.

Cross-reactions of the monoclonal antibodies raised against progesterone 11α-hydroxy-hemisuccinate are shown in Table 1. The concentration of the antibody was adjusted so that 80 to 90% of the available labeled progesterone was bound under similar experimental conditions to those used for the detection of the progesterone antibody (final incubation volume 500 μl). The decrease in the percentage of bound tritiated progesterone was then measured in the presence of increasing amounts of unlabeled progesterone or test steroids. The cross-reaction of the antibody was calculated as the ratio (expressed as a percentage) of the mass of progesterone to that of the test steroid

required to reduce the binding of tritiated progesterone by 50%[1]. The highest degree of cross-reaction with progesterone for antibody raised against the progesterone 11α-hydroxy-hemisuccinate was observed. Cross-reaction with 11α-dehydrocortisone was highest (2.179%) among other steroid hormones. Supernatants of clone PrM showed a remarkable specificity in their reaction against steroid hormones. The monoclonal antibody was compared with a rabbit antiserum raised against the same antigen, by conventional methods and used at a final dilution of 1:40,000. The properties of this rabbit antiserum were similar to those described in the literature[2]. Specificity of the monoclonal antibody for progesterone was higher than that of rabbit antiserum.

The RIA of progesterone using the supernatant of clone PrM was carried out as previously described[1]. For testing the titer of antibodies, an aliquot was thawed and diluted from 1:100 to 1:100,000 with barbital buffer (0.7 M, pH 9.6; final dilution 1:500 to 1:500,000). After the addition of tritiated progesterone to aliquots (250 μl) of monoclonal antibodies, samples were incubated for 30 min, at room temperature. Following incubation by shaking, 250 μl of 50% ammonium sulfate was added. After incubation (10 min, room temperature) and centrifugation (10 min, 3,000 rpm), the amount of tritium was measured in 200 μl of the decanted protein-free supernatant, as described previously[2]. A standard curve using monoclonal antibody (PrM) was compared with one using a rabbit antiserum raised against the same antigen (Fig. 1). Based on Scatchard Analysis the association constant (K_A) of the monoclonal antibody was 2×10^{10} and that of the rabbit antibody was 2×10^{9}.

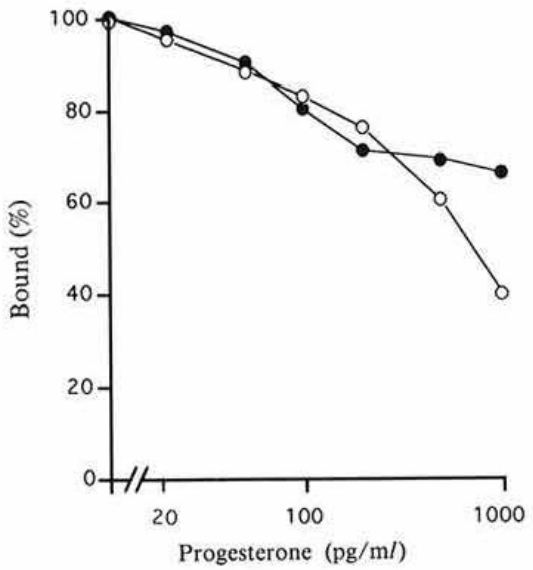

Fig. 1. Calibration curves using monoclonal antibody (PrM) (○) and a rabbit antiserum (●)
Tritiated progesterone (10,000 cpm) was used in a final assay volume of 500 μl. Highly specific monoclonal antibodies (1:10,000 dilution) and antisera (optimal dilution, 1:2,000) against progesterone 11α-hydroxy-hemisuccinate were prepared for RIA.

The method for estimating the level of progesterone has been previously described based on the principle of competitive displacement[1,2]. Compared to polyclonal antibodies, monoclonal antibodies are more suitable because the latter enable to supply a large quantity of specific immunological reagents by growing cloned hybridoma cells. Although a large amount of hybridoma cells must be grown *in vitro* to produce a specific antibody, extensive use in various assays is possible once the hybridoma cells are obtained.

In RIA or ELISA for progesterone, a highly specific antiserum is required since it enhanced the reliability, accuracy and practicality of the assay[4]. Under our experimental conditions, the binding activity of monoclonal antibodies exceeded that of polyclonal antibodies. Nevertheless, as an assay for determining the concentration of plasma progesterone the preliminary nature of this report must be emphasized since other immunoreactive progesterone preparations may have induced artifacts in the assay. The solid phase RIA and ELISA approaches are

applicable to steroids and other physiologically active low molecular weight compounds, which are now available in radio-active or enzymatically labeled forms with high purity.

The potential use of monoclonal antibodies in basic research is considerably high though much less widely discussed than more immediately obvious applications. Such monoclonal antibodies have already been widely used in studies on basic enzymology, nucleic acid structural analysis, and hormone receptor analysis. It is a normal practice currently for any group working on a hormone to both clone the genes coding for it and prepare monoclonal antibodies against it. One field of research in which monoclonal antibodies may prove to be of particular value is in studies on chromosomal proteins. The identification of chromosomal proteins which are responsible for determining hormone-producing cells has been particularly difficult. Monoclonal antibodies are comparatively an ideal tool for the separation of a complex mixture of proteins. As hybridoma production becomes a more routine laboratory technique, it is likely that this aspect of application will be expanded considerably.

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