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Characteristics and application of monoclonal antibody to progesterone

II. Development of progesterone enzyme-linked immunosorbent assay (ELISA)

Chung-boo Kang, Jong-shu Kim
College of Veterinary Medicine, Gyeongsang National University
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Progesterone의 단크론성 항체에 관한 특성 및 활용에 관한 연구

Ⅱ. ELISA 기법의 개발

姜　正　夫·金　鍾　秀
慶尙大學校 獸醫科大學 獸醫學科
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초록 : Progesterone의 단크론성 항체를 생산, 이용하여 감도가 높으면서도 신속히 측정할 수 있는 ELISA 기법을 처음으로 개발코저 실시하였다.

단크론성 항체는 종래의 면역방법에 의해 획득한 항혈청에 비해 약 10배의 결합율을 보였고 titer 역시 높았다. Dot-blot 분석 결과 단크론성 항체는 IgM이었다.

경합반응은 2시간으로 충분하였고, progesterone 표준용액을 이용한 표준 곡선은 0~1000pg/well 에서 거의 직선적이었다.

Progesterone의 단크론성 항체를 이용한 ELISA는 임상적으로는 물론 연구용으로도 신속한 황체의 기능 측정에는 물론 각종 번식 관련의 지표로 충분히 활용될 수 있을 것으로 판단된다.

Key words: Monoclonal antibody, progesterone, enzyme-linked immunosorbent assay(ELISA), cattle.

Introduction

Nonreturns to estrus or reproductive tract palpation by skilled personnel have been the common methods used for pregnancy diagnosis in cattle. The inability of dairymen to detect estrus and the delay of 45 to 60 days for palpation are the primary disadvantages of these methods.

Thus a simple, accurate, early pregnancy diagnostic technique would be advantageous for dairy industries.

Since the availability of RIA,[1,2] progesterone levels have become one of the major hormonal parameters used to monitor reproductive status in cattle.

The most popular assay for this purpose has been using tritiated progesterone as label, but an alternative RIA using progesterone labelled with ^{125}I has been propose.[3,4]

While both of these methods can give accurate and reliable results they suffer from the problems

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associated with the use of radioisotopes, e.g. short half life of label (^{125}I) cost of scintillators and radioactive waste disposal (^{3}H, ^{14}C) and restriction to institutes which have permission and facilities to handle radioisotopes.[4,5]

Such problems have prompted the search for alternative labels for use in immunoassays.

Proposed alternatives include: enzymes, erythrocytes, bacteriophages, fluorescent groups and stable free radicals.

Of these labels, enzymes have received by far the greatest attention assays with sensitivity, reproducibility and practicability similar to those of RIA.[6-8]

Most steroid enzyme immunoassays(EIA) currently available use liquid-phase double antibody separation or double-antibody solid-phase in which bound conjugate is separated from the free fraction by reaction with anti-immunoglobulin covalently linked to cellulose, or the simple solid-phase method in which separation of bound from free is achieved by precipitation of primary antibody coupled to test tubes or polystyrene beads.[9-12]

These methods consume considerable time and reagents becomes of the requirement for several washing and centrifugation steps involving the reactants.

Because of these several problems, we developed an effective enzyme-linked immunosorbent assay (ELISA) system instead of EIA that would be equal or superior to RIA for the assay of progesterone,[13,14] and also we have been produced monoclonal antibodies to progesterone.[15]

Monoclonal antibodies are higher specific for the polypeptide hormones than conventional polyclonsl antibodies.

However, no report for ELISA of progesterone using the monoclonal antibody to progesterone has been available.

This report describes the development of a sensitive, rapid, solid-phase microtitre plate assay using the monoclonal antibody to progesterone.

Materials and methods

Reagents: Horseradish peroxidase(HRP) labelled progesterone, bovine serum albumin (BSA, Cohn fraction V), sodium azide, 2, 2′-azino-di-(3-ethylbenzthiazoline sulphonic acid) diammonium salt (ABTS), Tween 20, EDTA acid (tetrasodium salt) and citric acid (anhydrous) were purchased from Sigma Chemical Co. (U.S.A.).

Flat-bottemed polystyrene 96-well microelisa plates (Immulon 2) were purchased from Dynatech Laboratories, Inc. (Alexandria, Virginia), and 8 and 12-channel variable-volume Titertek pipette and plate sealer covers were obtained from Flow Laboratories, Inc. (Inglewood, California). All plates were read in Dynatech MR 700 automatic microelisa plate reader (Dynatech Laboratories, Inc., U.S.A.)

Preparation of antiserum (polyclonal antibody, PCA) : Antiserum againt progesterone 11α-hydroxy-hemisuccinate-BSA was produced in three male New Zealand White rabbits, 3~5 month old, by the immunization protocol as described previously.[13]

Each rabbit received 1mg (1mg/ml) of the emulsified immunogen at 0, 2 and 4 weeks with a booster (1mg) administered approximately every 3 weeks was thereafter until no further increase in titer was obtained. Rabbits produced excellent antisera by day 90, and one antiserum which bound 30% of the titrated label in the absense of added steroids at a dilution of 1 : 2500 was chosen for use in the RIA and ELISA.

Production of monoclonal antibody (MCA) : Monoclonal antibody against progesterone 11α-hydroxy-hemisuccinate was produced using BALB/c mice as described previously.[15] The antigen conjugated to bovine albumin (Sigma Chem. Co. U.S.A.) was dissolved in 0.9% physiological saline solution and mixed with an equal volume of Freund's complete adjuvant Difco Lab., U.S.A.) and 50, 100μg of antigen were given as an intraperitoneal injection to each 3 female BALB/c mice.

The cell fusion was used the modified method to generate hybrid cell lines producing antibodies to progesterone.[16]

The cell line used for fusion was P3-X63-Ag8-Ul (P3U1).

Antibody assessment: The screening of antiserum against progesterone 11α-hydroxy-hemisuccinate-BSA and monoclonal antibody activities to progest-

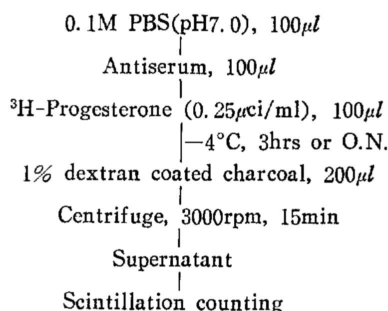

Fig 1. Progesterone antibody titer determination for PCA and MCA by RIA

erone were principally carried out using conventional RIA as shown in Fig 1.

In addition, antibody activities were also performed by ELISA.

Stock solutions: The followig stock solutions gave optimal results for the ELISA system: (1) coating buffer: 0.05M-sodium bicarbonate, pH9.6; (2) washing buffer: 0.15M-NaCl containing 0.05% Tween 20; (3) assay buffer: 0.1M-Sodium phosphate, pH7.0, containing 0.87% NaCl and 0.1% BSA; (4) substrate: 250μl 40mM-ABTS and 80μl 0.5M-H_2O_2 were added to 24.67ml 0.05M-citrate (pH4.0), no more than 15 minutes before use on the day of assay; (5) ABTS stopping reagent: 25ml stopping solution containing 25.0μl 1.0M-EDTA and 25ml 0.15M-hydrofluoric acid containing 0.006M-NaOH.

ELISA procedure: Flat bottomed polystyrene microtitre plate (96 well) were coated with 50μl per well of BSA-absorbed rabbit anti-progesterone gamma globulin and monoclonal antibody to progesterone in coating buffer at a dilution of 1 : 5000 except for the first row.

The plate was sealed tightly with a water proof plate-sealer cover and incubated usually overnight at 4°C, although plate could be used in as little as 4 hours or could be stored at 4°C for several weeks in the unwashed or washed state. To remove antiserum not bound to polystyrene, the wells of the plate were emptied by inversion washed five times with wash solution, drained and dried inverted on paper towels.

Before the assay, 125μl assay buffer was added to each tube, and 50μl aliquots were added to the wells in duplicate.

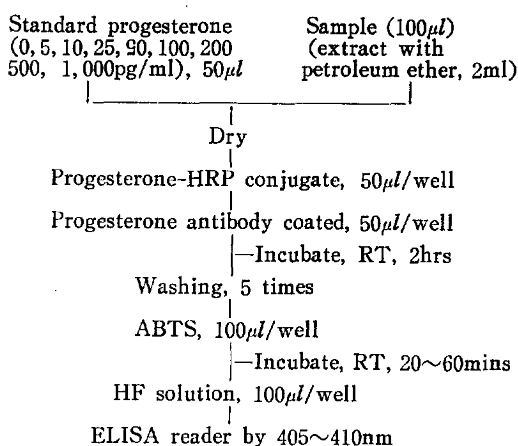

Fig 2. Enzyme-linked immunosorbent assay for progesterone.

For the standard curve, 50μl of progesterone standards was used (nine well standard, 0~1000Pg). This was followed immediately by 50μl of the diluted progesterone-HRP conjugate in assay buffer to initiate a competition reaction.

Immediately after the addition of conjugate, plates were covered and incubated for exactly 2 hours at room temperature.

Separation of free from bound progesterone was achieved by emptying the plate and washing five times.

The outline of following steps for ELISA procedure was shown in Fig 2.

A standard dose-response curve was constructed by plotting percent bound against amount of progesterone added using logistic curve-fitting program.

Result

Antiserum assessment: No change in binding characteristics or titre of the antiserum as determined by RIA was observed after adsorption with BSA on PCA.

Fig 3. Shows a dose-response curve by RIA when a final dilution of antibodies (1 : 10,000) was used for coating.

When the percent bound radioactivities were plotted against the logarithm of the dose of progesterone, this assay was linear from 10 to 200 picograms.

A substantial increase in absorbance readings was

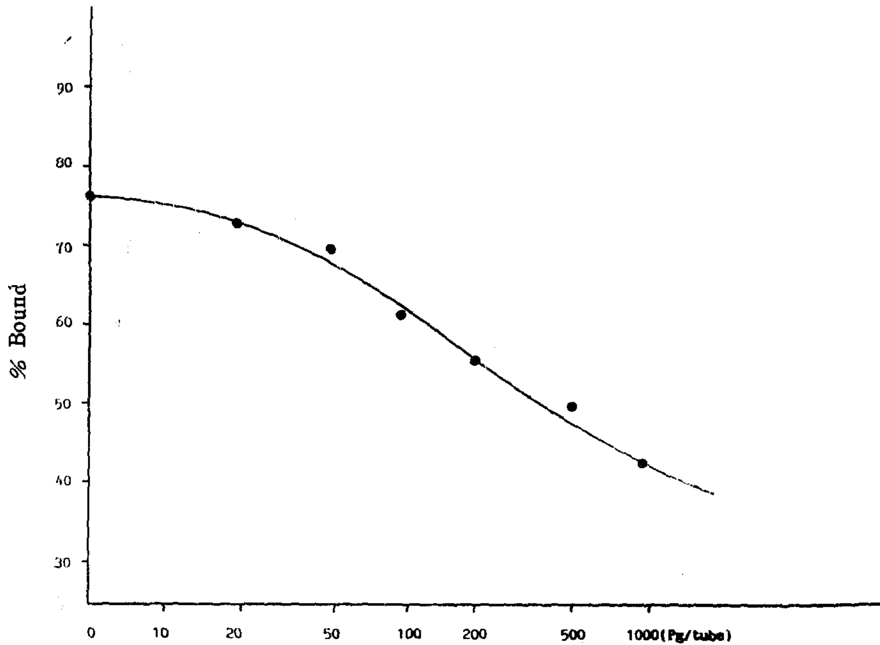

Fig 3. Radioactivity bound to progesterone polyclonal antibody diluted to 5×10^{3}

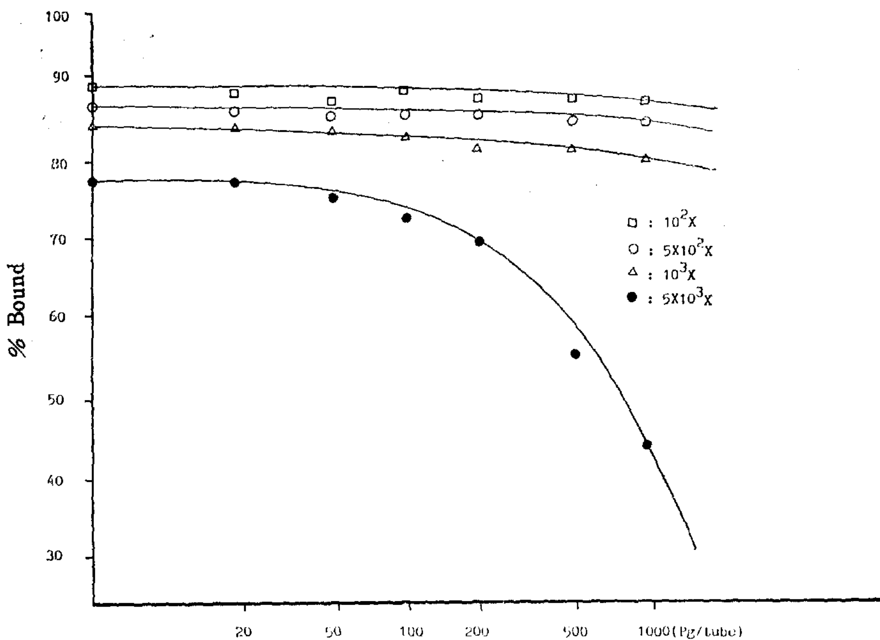

Fig 4. Radioactivity bound to progesterone monoclonal antibody

obtained for the progesterone 11α-hemisuccinate-HRP conjuganate after adsorption of the anti-BSA antibodies.

Monoclonal antibody to progesterone was much more binding about 10 times and titre efficiently than polyclonal antibody. The coefficient of variation calculated for each point was less than 5% (Fig 4).

Monoclonal antibody coating was much more efficient radioactivity bound to progesterone as compared the results in Fig 1.

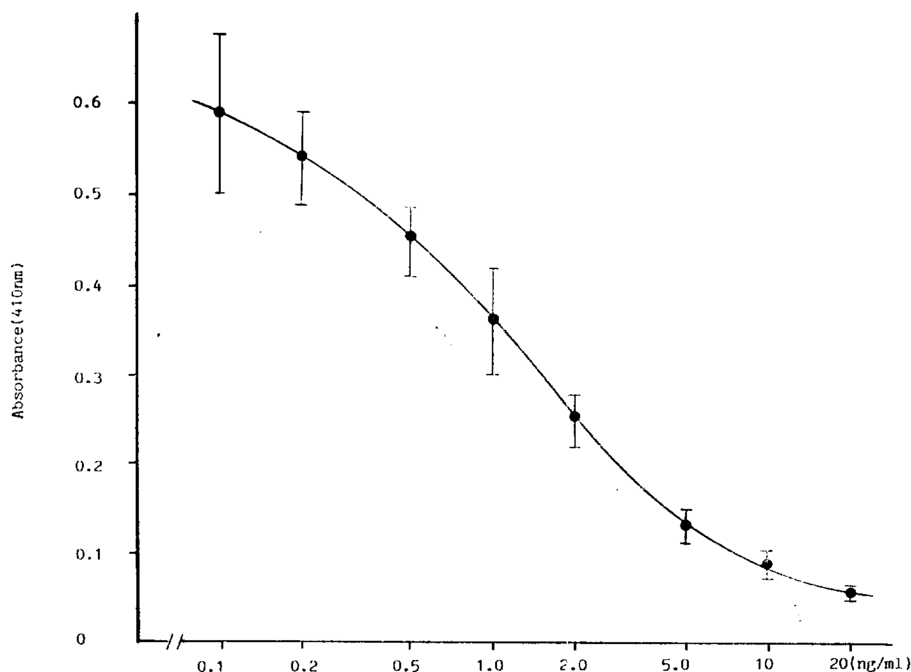

Fig 5. Standard curve for enzyme-linked immunosorbent assay of progesterone.

Dot-blot analysis of monoclonal antibody revealed a single precipitation band when reacted with rabbit anti-mouse IgM and anti-mouse K (Bethyl Lab., Texas, U.S.A.). No precipitation band was produced with anti-mouse IgG_1, IgG_2, IgG_3 and λ.

Enzyme label: The conjugation reaction was 95% efficient.

Up to 78% of the enzyme activity in the conjugate preparation could be bound by excess antiserum.

The progesterone 11α-hemisuccinate-HRP and progesterone 3-CMO-HRP conjugates were used for the solid-phase antibody dilution. Less sensitivity was obtained when the same chemical bridge, homologous system (11α-hemisuccinate conjugate). The use of the heterologous system involving two different chemical bridges (hemisuccinate for immunogen and 3-CMO for conjugate) conferred considerably greater sensitivity to the ELISA.

Incubation intervals: In the competitive reaction between enzyme conjugated and free progesterone for sites on the solid-phase antiserum, equilibrium was not reached within 3 hours. However, the rate of change at 2 hours ($p<0.1$ absorbance units/h) was relatively low.

To ensure that excess substrate was available during the entire reaction interval, the end Product was assayed after stopping the reaction at 60 minutes.

Standard curve: An example of standard curves for progesterone ELISA is shown in Fig 5.

In routine use, a monoclonal antibody dilution of 1 : 5,000 to coat the wells and a conjugate(3-CMO) dilution of 1 : 25,000 gave consistent optical density ranges of 0.6~0.7(Eo. 0 pg progesterone) to 0.06~0.07(1,000pg progesterone) at 410nm. The standard dose-response curve was linear from 0.1 to 10ng/ml (Fig 5.).

Discussion

The micro ELISA test is based on the principle of the competitive reaction for detection of small antigens.[17]

The competitive type of assay is the only one that has been used for steroid determination, since steroid molecules are too small (e.g. progesterone, 314.4) to bind two antibody molecules as required in the sandwich assay technique.[18]

The only other microtitre plate EIA descriaed for progesterone is an homologous assay with a sensit-

ivity of 5.0pg/well and a limited change in absorbance over the full range of the standard curve.[19]

The removal of antibodies not specific to assay is important in the development of a sensitive solid phase EIA microassay system.

This is because the presence of a large subset of non-specific antibodies limits the number of specific progesterone antibodies which can be bound on to the small polystyrene well surface.

Results in significantly reduced binding of the progesterone molecules present(either in the free or conjugated form) to the covalently adsorbed progesterone antibody and accounts for the lower absorbance readings and sensitivity. However, method of determining progesterone have been previously described using the principle of competitive displacement, polyclonal antibodies were used.[6~14]

Monoclonal antibodies have an advantage that they offer a means of preparing a large amount of specific immunological reagents by growing cloned hybridoma.[15] Althogh large amount of hybridoma must be grown in vitro in order to produce a specific antibody, once it is obtained it may be used for thousands of assay.

Monoclonal antibodies must be standardized by rigorous cross-reaction studies prior to use an analytical reagent since the reliability of the assay is dependent on the specificity of the antibody used.

In RIA or ELISA for progesterone highly specific antiserum is desirable since it improves the practicability of the assay.

The monoclonal antibody to progesterone used in this assay is highly specific for progesterone, having a minimal cross reaction with other progesterone tested.[15]

Nevertheless, as an assay for progesterone the preliminary nature of this report must be emphasized since other immunoreactive progesterone contribute to the assay.

The solid phase ELISA approach is applicable to evaluation of appropriate timing of insemination during assumed estrus, confirmation of return or of non-return of estrus, monitoring of cyclicity during post-parturient period, monitoring therapautic measures, and monitoring programmes such as those with embryo transfer experiments.[20~27]

This ELISA system is a simple, rapid, and sensitive, so this may be advantageously used to study of luteal function and reproductive status.

Summary

This experiment was carried out to develop a sensitive, rapid, solid-phase microtitre plate assay of progesterone using the monoclonal antibody to this hormone.

Monoclonal antibody to progesterone was much higher titre and binding affinity about 10 times than conventional polyclonal antibody to progesterone.

Dot-blot analysis of monoclonal antibody revealed a single precipitation band when reacted with anti-mouse IgM and anti-mouse K.

A competitive reaction was used with a reaction time of 2 hours.

The standard dose-response curve was linear through 1,000pg/well.

This ELISA system approach is applicable to evaluation for the rapid assessment of luteal function and reproductive status in both clinical and research in a wide variety of species.

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