Production of Monoclonal Antibodies Specific for Progesterone, Estradiol by Simultaneous Injection of Different Steroids

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Abstract: We report here the development of hybrid cells producing monoclonal antibodies specific for two different steroid hormones with mixed immunization using hybridoma technology. BALB/c mice were immunized with a mixture of three steroid antigens: progesterone, estradiol and testosterone linked to bovine serum albumine. These mice were used for fusion. In the two fusion experiments, ELISA tests showed that among 645 wells only 2 hybrids reacted with progesterone (MAM 3C2, MAM 3E3) and one of them reacted specifically with estradiol (MAM 7H7). Three types of monoclonal antibodies were detected: IgG1, IgG2a and IgG2b and the affinities of these antibodies were found to be $10^{-8}$–$10^{-9}$ M. Monoclonal antibodies did not cross react with the other steroid hormones.

Key Words: Immunodiagnosis, hybrid cell, monoclonal antibody, steroid hormones, ELISA.

Introduction

The introduction of monoclonal antibodies has contributed a great deal to the immunodiagnostic area in the past 20 years. Although production of monoclonal antibodies against several antigens was highly successful, the same accomplishment couldn’t be achieved in producing efficient and specific monoclonal antibodies against steroids. Attempts have been made to solve this problem by increasing the amount of antigens, yet the problem of specificity...
still needs to be overcome (1). Thus, using different immunization regimens and the antigens 6β-hydroxyprogesterone hemisuccinate and 11α-hydroxyprogesterone hemisuccinate conjugated to bovine serum albumine (BSA), Fantl et al. (2-4) have produced 35 monoclonal antibodies against progesterone with a wide range of specificities and affinities ($K_a$: 8x10^{-2}-3x10^{10} M^{-1}). Simultaneous production of monoclonal antibodies to mixtures of different steroid antigens linked to BSA have been investigated (2). Only six antibodies were developed against progesterone in the first fusion experiment despite the relatively high binding to this steroid shown by the mouse serum. However, in the second fusion experiment, relatively few monoclonal antibodies were developed against these antigens.

Several types of antibodies can be produced by activating the immune system with different antigens simultaneously (5, 6).

In this study a multiple-immunization method was applied in order to obtain monoclonal antibodies with high specificity against different steroids (namely progesterone, estradiol and testosterone) and three monoclonal antibodies showing high specificity against two of the antigens (progesterone and estradiol) were recovered.

This report describes the development of a hybridoma line producing high affinity monoclonal antibodies against each steroid using this multiple-immunization technique.

**Materials and Methods**

**Reagents**

Progesterone 3(0-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-(0-carboxymethyl) oxime conjugated to bovine serum albumin (BSA.E) (steroids: BSA, 32:1), BSA. testosterone (BSA.T), BSA. aldosterone (BSA.A), BSA. corticosterone (BSA.C), and dexamethazone were obtained from SIGMA. Dulbecco’s Modified Eagles Medium (DMEM), penicillin-streptomycin (antibiotics), hypoxanthine, aminopterine, thymidine (HAT) and hypoxanthine, thymidine (HT) were purchased from GIBCO.

**Immunization**

BALB/c mice were immunized with a mixture of three steroid antigens: progesterone, estradiol and testosterone linked to bovine serum albumine. A solution containing these antigens (containing 10 µg of each antigen) was prepared in phosphate buffered saline (PBS: 10 mM K$_2$HPO$_4$, 10mM KH$_2$PO$_4$, 0.15 M / L NaCl, pH 7.2) and mixed with equal volumes of Freund’s complete adjuvant (SIGMA) and 0.2 ml of this complex was given by intraperitoneal injection to five eight-week old BALB/c mice. Booster injections were given with three weekly intervals using Freund’s incomplete adjuvant (SIGMA). Four days before fusion, an intravenous injection of 5 µg of each steroid antigen in 0.1 ml PBS without adjuvant was administered.
Selection of Spleen Donors

The selection of mice for fusion was based on the antibody titer measured in the tail blood samples. The blood was collected in a microfuge tube containing sodium citrate (Merck). The tubes were centrifuged at 6000 rpm /min to remove red blood cells. The plasma samples (2 µl) were tested with each antigen using the ELISA test system. Plasma (2 µl) from a nonimmunized mouse in PBS (100 µml) was used as the control. The mice which had the highest antibody activity to progesterone, estradiol and testosterone were selected as the spleen donors for fusion.

Fusion

F0 the myeloma cells line (ATCC CRL 1646) was hybridized with immune spleen cells using polyethylene glycol 4000 as fusing agent (Merck) (7-10). After fusion, the cells were distributed into the 96 well culture plates (NUNC) at a 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 16mM 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 nonimmunized BALB/c mouse to assist the early stages of growth. Positive hybrid clones producing antibodies with the highest specificity were subcloned by dilution methods (11). At each stage of growth, aliquots of the hybrid cultures (3-5.10^6 cells) were transferred into 80% DMEM, 20% FCS and 10% dimethylsulphoxide (Merck) and frozen in liquid nitrogen.

Elisa

The indirect enzyme-linked immunosorbent assay (ELISA) (12-15) was used for the screening of the hybridoma supernatants for anti-progesterone, anti-estradiol and anti-testosterone. 96 well polystrene plates (NUNC immunoplates) were coated with 200 ng BSA.P in parallel with BSA.E, BSA.T in 100µl 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 (Merck) in PBS). Then, 0.2 % milk powder in 200µl PBS was added to the wells and the plates were incubated for 1 h at 37 °C followed by three washings. Each hybridoma supernatant 100µl was tested with each steroid (progesterone, estradiol and testosterone). 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 (in 100µl 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, 1mM MgCl, 0.1 M glycine pH 10.4) containing 1mg per ml para-nitrophenyl phosphate (Merck) was added to each well (100µl) and incubated for 45 min at room temperature in darkness. The results were obtained by measurement of the optical density at 405 nm.

Purification of Monoclonal Antibodies and Subisotyping

Monoclonal antibodies were purified from the hybridoma supernatant by ammonium sulfate (Merck) precipitation at between 30 and 50 % saturation. The pellet dissolved in 10 ml PBS was dialyzed against 1000 ml of PBS for 24 hours at 4 ° C and submitted to immunoaffinity
chromatography using solid-phase bound protein G (S. aureus) as the IgG-immunotrap (Mab-Trap/Pharmacia) (16). Ig typing of the monoclonal antibodies was performed using a hybridoma subisotyping kit based on enzyme immunosassay (ELISA) (Boehringer Mannheim).

**Affinity Measurements**

Affinity measurements of the monoclonal antibodies were carried out by equilibrium dialysis using radiolabelled (3H) progesterone (NEN, specific activity: 109.5 Ci /mmol) and (3H) 17β-estradiol (NEN, specific activity: 87 Ci /mmol). Antigens and antibodies were incubated in equilibrium dialysis cells for 20 h at room temperature on a shaker. Radioactivity was measured using an LKB (Wallac) 1212 Rackbeta Liquid Scintillation Counter. The resulting slope was calculated using The GraFit program and dissociation constants were determined according to the method of Scatchard (17-19).

**Results**

**Response to Immunization and Selection of Spleen Donors**

Figure 1 shows the binding of each steroid hormone (progesterone, estradiole and testosterone) to blood samples taken from mice (2 weeks after they had received booster injections, i.e 3, 6 and 9 weeks after the initial immunizations). Two of the five immunized mice were selected as spleen donors for fusions.

**Cell Fusion and Antibody Production**

Mice number one and two showing the highest antiserum titers for all the three steroid hormones were used for the fusions. The results of the fusions following immunization based on the use of a mixture of the three steroid antigens are summarized in Table 1.

In the first fusion experiment hybrid cells were screened in 396 wells and supernatants from 21 of the 396 wells showed steroid-binding activity. One, MAM-3C2, was proved to be progesterone specific, another one, MAM-7H7, was proved to be estradiol specific. In the second fusion experiment 249 hybrids were obtained from 672 wells. Supernatants from 8 of the 249 wells showed steroid-binding activity and only one, MAM-3E3, was proved to be progesterone-specific.

Table 2 shows the clones producing antibodies with high affinity and specificity for BSA.P and BSA.E.

**Characterization of Antibodies**

Two progesterone-specific monoclonal antibodies (MAM3C2, MAM 3E3) and one estradiol (MAM 7H7) were obtained. Monoclonal antibodies were identified as the IgG class which were IgG2a, IgG2b and IgG1 subtype using a hybridoma subisotyping kit system. They also contained kappa light chains. The affinity of these monoclonal antibodies for progesterone and for estradiol was determined by equilibrium dialysis. The affinity constants (Kd,M) measured for the purified monoclonal antibodies are shown in Table 3.
Discussion

We used the method of Köhler and Milstein (10) to generate hybrid cell lines producing highly specific antibodies. Mice were simultaneously immunized with three different steroid antigens. As seen in Figure 1, the antibody response to these steroid antigens showed wide
variation and the mice which had the highest antibody activity to progesterone, estradiole and
testosterone were selected for the fusion experiment. The mixture of three different steroid
antigens was used to immunize the mice in the expectation that at least one high affinity
antibody to each antigen might be obtained. By using hybridoma technology, we developed
hybrid cells producing antibodies for the estradiole (MAM 7H7) and for the progesterone
(MAM 3C2, MAM 3E3) in two fusion experiments. These monoclonal antibodies did not cross
react with other steroid hormones using indirect - ELISA. The affinities of these antibodies
were found to be $10^{-8} - 10^{-9}$ M. We couldn’t obtain monoclonal antibodies against testosterone.

The weak immunogenecity of low molecular weight steroid hormones has been reported
(6). To overcome this handicap mixed immunization trials were performed by Fantl and Wang.
In this study we described a method with the ultimate aim of developing immunodiagnostic
system for the different steroid hormones (2-4). Our results are compatible with the other
monoclonal antibody studies reported in the literature (2).

Using this immunization method, it was possible for a single spleen donor to produce an
optimal response to three antigens simultaneously. We produced monoclonal antibodies (MAM
3C2, MAM 3E3, MAM 7H7) against two antigens, progesterone and estradiole.

These monoclonal antibodies can be used efficiently in the early and sensitive detection of
progesterone and estradiole by immune diagnostic test systems (i.e., ELISA).

<table>
<thead>
<tr>
<th>Steroids used in ELISA</th>
<th>Hybrids</th>
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<tbody>
<tr>
<td></td>
<td>MAM-7H7</td>
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<tr>
<td>Progesterone</td>
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<tr>
<td>Estradiole</td>
<td>1.14</td>
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<tr>
<td>Testosterone</td>
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<td>Dexamethazone</td>
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<td>BSA</td>
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<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
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<th>Immunoglobulin class</th>
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<tr>
<td>MAM 7H7</td>
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<td>IgG1</td>
</tr>
<tr>
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<td>$3.1 \times 10^{-9}$</td>
<td>IgG2a</td>
</tr>
<tr>
<td>MAM 3E3</td>
<td>$2.4 \times 10^{-8}$</td>
<td>IgG2b</td>
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Table 2. Comparison of the reactivity (OD$_{405}$) of the monoclonal antibodies with different steroids.

Table 3. Apparent dissociation constants ($K_w$) and immunoglobulin class of monoclonal antibodies obtained using immunization with BSA.P+BSA.E+BSA.T
References


