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Development and Immunochemical Properties of Two Monoclonal Antibodies Specific to Human Chorionic Gonadotropin

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Using a hybridoma technique, spleen cells of Balb/c mice immunized with human chorionic gonadotropin (hCG) were fused with NS-1 mouse myeloma cells. Two hybrid cell lines, clones KS-8 and KS-19, secreting monoclonal antibodies to hCG, were isolated. KS-8 and KS-19 belong to the immunoglobulin G_1 subclass. With the aid of a double-antibody radioimmunoassay, it was established that the KS-8 monoclonal antibody recognizes an immunodeterminant of the β-subunit of hCG, whereas the KS-19 monoclonal antibody recognizes an epitope present on the α-subunit of hCG. The KS-8 monoclonal antibody specifically reacts with human chorionic gonadotropin and shows cross-reactivity of less than 0.3% to other related human glycoprotein hormones. On the other hand, using a hemagglutination test based on antibody-induced agglutination of sheep red blood cells coated with hCG, it was shown that only the KS-19 monoclonal antibody was capable of inducing a positive reaction, although both monoclonal antibodies had similar binding capacity to the coated cells. The results from the dual screening procedures demonstrate that KS-8 and KS-19 monoclonal antibodies show high sensitivity in two different assays, and are hence useful for the qualitative and quantitative determination of hCG by both radioimmunoassay and hemagglutination inhibition tests.

Keywords: Human chorionic gonadotropin (hCG), Monoclonal antibody.

Introduction

Oncofetal glycoprotein hormone, human chorionic gonadotropin (hCG) is produced by the trophoblast from early pregnancy. This placental hormone serves to maintain the steroid secretion of the *corpus luteum* (Talwar *et al.*, 1980). hCG is secreted into serum and urine, not only in normal pregnant women but also in patients with choriocarcinoma. Hence, the detection of hCG in biological fluids has been used as tools for the diagnosis of pregnancy and choriocarcinoma for many years (Sexena *et al.*, 1974; Catt *et al.*, 1975; Braunstein *et al.*, 1976).

Human chorionic gonadotropin consists of two nonidentical α- and β-subunits (Morgan and Canfield, 1971; Vaitukaitis *et al.*, 1976) and contains about 30% carbohydrates (Mizuochi *et al.*, 1983). Among the six components of the carbohydrate moiety, N-acetylneuraminic acid is essential to the biological activity of the hormone (Schwartz and McCormack, 1972). The sugar chains of normal hCG were mostly sialynated, while the chains of choriocarcinoma hCG were free from sialic acid (Mizuochi *et al.*, 1983). The α-subunit is interchangeable with other pituitary glycoprotein hormones, whereas the β-subunit is responsible for biological and immunological specificities (Jensen and DeSombre, 1972; Schwartz and McCormack, 1972; Vaitukaitis *et al.*, 1976). In particular, the 113 N-terminal amino acids sequence of β-human luteinizing hormone (β-LH) has more than 80% homology with the 113 N-terminal amino acids sequence of β-human chorionic gonadotropin (β-hCG). However, β-LH lacks C-terminal segment of another 32 amino acids specific to β-hCG (Carlsen *et al.*, 1973; Morgan, *et al.*, 1975).

Clinical assays for the diagnosis of pregnancy are based on anti-hCG antibodies. Since secretion of hCG is still at relatively low levels by the seventh wk of pregnancy, radioimmunoassays are preferred to the hemmaglutination test for the early diagnosis of pregnancy. The development

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of specific and sensitive antibodies against hCG suitable for radioimmunoassays has been a major subject of many investigators (Chen *et al.*, 1976). Although a number of polyclonal antisera have been obtained against β-hCG, they had varying degrees of cross-reactivity to human LH and fairly high specificity to hCG (Salahuddin *et al.*, 1976; Vaitukaitis *et al.*, 1976). Because of their unique specificity, several hybridoma cell lines secreting monoclonal anti-hCG antibodies were developed (Kohler and Milstein 1975; Miggiano *et al.*, 1980; Bottger *et al.*, 1993; Krichevsky *et al.*, 1994; Jeyakumar *et al.*, 1997; Fotinou *et al.*, 1998) using the cell hybridization method (Choi, 1993; Lee *et al.*, 1994; Ha *et al.*, 1997; Lee and Jang, 1998). Monoclonal antibodies do not usually induce precipitation reactions against their appropriate antigens (Milstein *et al.*, 1980). Mixtures of different monoclonal antibodies, however, with each antibody directed against different epitopes of the corresponding antigen can induce the formation of an antigen-antibody precipitating complex (Howard *et al.*, 1979; Slaughter *et al.*, 1980). A critical factor to the usefulness of a monoclonal antibody is the antibody affinity, that characterizes the interaction between the monoclonal antibody and its corresponding antigens (van Erp *et al.*, 1991). In this study, we report the development of two monoclonal antibodies to hCG and immunochemical properties that recognize two different epitopes of the α- and β-subunits of the molecules.

Materials and Methods

Materials Purified human chorionic gonadotropin was obtained from Calbiochem-Novobiochem (La Jolla, USA), free Na ^{125}I (specific activity 13-17 mCi) from Amersham (Picataway, USA) and ^{125}I-human chorionic gonadotropin from New England Nuclear (Boston, USA). Purified α- and β-human chorionic gonadotropin was obtained from Boehringer Mannheim (Indianapolis, USA) and purified human luteinizing hormone from Pentex (Santa Clara, USA). All other chemicals were of analytical reagent grade.

Immunization Four to six wk old female Balb/c mice were immunized with an intraperitoneal injection of 250 IU of purified human chorionic gonadotropin in complete Freund's adjuvant. Boosters were given at three wk intervals and fusion between spleen cells of the immunized mice and a P3-X63-Ag. 8 (NS-1) mouse myeloma cells was performed 3 d after the second booster.

Cell hybridization and cloning Hybridization between immunized spleen cells and NS-1 myeloma cells at a ratio of 10:1 was induced by exposure to polyethyleneglycol, 1500 MW, for 1 min by the method of Galfre *et al.* (1977). Fusion cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated horse serum containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterine and 1.6×10^{-5} M thymidine (DMEM-HAT medium). Cultures were refed with DMEM-HAT medium every 3–4 d. Culture supernatants were screened for anti-human chorionic gonadotropin antibodies by a double antibody radioimmunoassay method on the 14th d post-incubation. Positive wells were isolated and cells were cloned by the limiting dilution method.

Double antibody radioimmunoassay The reaction mixture contained 100 μl of approximately-diluted monoclonal anti-human chorionic gonadotropin antibody in 0.01 M phosphate buffer, pH 7.8, containing 0.15 M sodium chloride, 0.1% sodium azide, and 0.5% bovine serum albumin and 100 μl of ^{125}I-human chorionic gonadotropin (specific activity 80 μCi/μg), with a total radioactivity of approximately 15,000 cpm/assay. The reaction mixture was incubated at 37°C overnight. After the incubation, 0.1 ml of 2% normal mouse serum diluted in buffer, 0.1 ml of 0.1 M EDTA in water, pH 7.8, and 0.1 ml of rabbit anti-mouse IgG antiserum at a concentration of 1 mg/ml buffer were added to the reaction mixture. Polyethylene glycol, 6000 MW, was then added to a final concentration of 3 mg/ml. The antigen-antibody complex was separated from free antigen by centrifugation and the radioactivity of the precipitate was determined with a Packard gamma-counter (Model Multi Prias 5302).

Preparation of ascites in mice Approximately 5–10 × 10^6 cultured cells of the selected hybridoma clones, KS-8 and KS-19, were injected intraperitoneally into pristane-treated Balb/c mice. Ascitic fluids of tumor-bearing mice were harvested 10–20 d following inoculation of the hybridoma cells and subsequently used for antibody characterization.

Determination of heavy-chain isotypes The immunoglobulin composition of the purified antibodies was determined by the immunodiffusion method, using class-specific anti-serum against mouse immunoglobulin.

Iodination of antibodies Purified monoclonal antibodies were obtained from ascitic fluids by precipitation with ammonium sulphate at 50% saturation. Fifty μg of monoclonal antibodies was iodinated with 1 mCi carrier-free Na ^{125}I (specific activity 13–17 mCi/μg) by the chloramine-T method (Howard *et al.*, 1979).

Direct binding and inhibition assay The binding characteristics of monoclonal antibodies were determined by binding and inhibition assays. The direct binding assay of the monoclonal antibodies was carried out as follows: Plastic microtiter plates were coated with monoclonal antibodies, 20 μg/well, for 24 h followed by washing with 1% bovine serum albumin. Different concentrations of human chorionic gonadotropin were added and incubated overnight at 4°C. Excess human chorionic gonadotropin was removed by extensive washing with bovine serum albumin. ^{125}I-labeled monoclonal antibodies at saturation levels (1×10^5 cpm-1 μCi/μg) were added to the bound human chorionic gonadotropin and incubated for an additional 24 h followed by extensive washing with bovine serum albumin. Wells were cut off and counted in a Packard gamma counter (Model Multi Prias 5302). Inhibition of binding monoclonal antibodies to human chorionic gonadotropin by monoclonal antibodies with different anti-human chorionic gonadotropin specificity was determined by solid phase

radioimmunoassay. Varying amounts of the unlabeled hormones competed with the ^{125}I-monoclonal antibody.

Hemagglutination test Sheep red blood cells were coated with a crude preparation of human chorionic gonadotropin using 5% glutaraldehyde. Dilutions of 0.1 ml of monoclonal anti-human chorionic gonadotropin and a polyclonal rabbit anti-hCG antibody were incubated with 0.1 ml of coated sheep red blood cells. Agglutination was observed after 60–120 min of incubation at 25°C. The antibody titer in this direct hemagglutination assay system was defined as the maximum dilution ratio of the antibody at which a positive agglutination was observed.

Results

Development and classification of antibodies Of the 288 wells plated after cell fusion, 98 grew single colonies of which 17 colonies were positive in the initial screening. Among these colonies, the best three were chosen for cloning at limiting dilution with 0.5–1 cell per well in 96-well plates. After the initial cloning, positive responses were obtained in only two wells, all of which were derived from the same initial colony. Two of these, KS-8 and KS-19, were cloned again at limiting dilution. An antibody titer of 1:240 was obstained for KS-8 after propagation in the tissue culture increased to 1:1200 after the recovery in ascitic fluid. The anti-hCG antibodies obtained were found to belong to the IgG_1 subclass (Fig. 1).

Characterization of monoclonal antibody produced by the KS-8 hybridoma clone Monoclonal antibody produced by the KS-8 hybridoma clone showed highly specific binding capacity to ^{125}I-labeled hCG and rare bindings to the other pituitary ligands (Fig. 2). The cross-reactivity of KS-8 monoclonal antibody with human luteinizing hormone, human follicle stimulating hormone, and human thyroid stimulating hormone was less than 0.3%, and cross-reactivity against the α-subunit of hCG was less than 1.2% (Table 1). The KS-8 monoclonal antibody failed to induce a positive hemagglutination reaction to sheep red blood cells coated with hCG (Table 2).

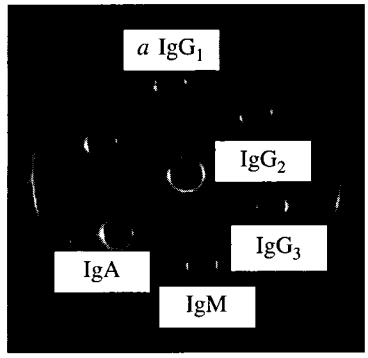

Fig. 1. Immunodiffusion test with the monoclonal antibody produced by the hybridoma clone.

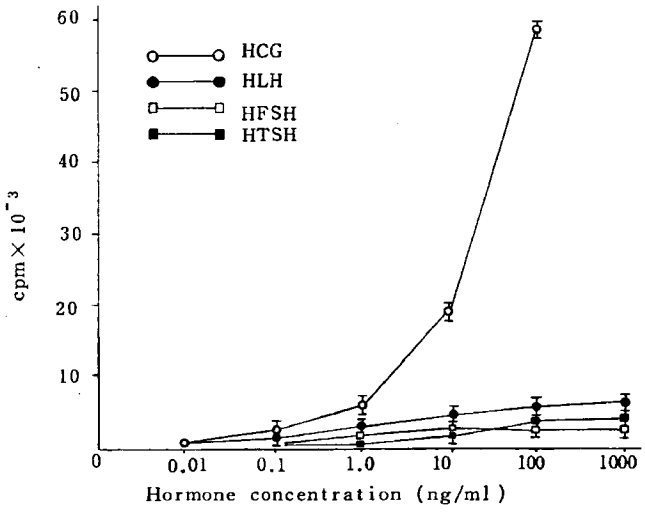

Fig. 2. Characterization of the specificity of KS-8 monoclonal antibody. The binding activity and specificity of KS-8 monoclonal antibody was determined by the solid phase radioimmunoassay in which plastic microtiter plates were coated with 20 μg purified KS-8 monoclonal antibody. Binding of different concentrations of human chorionic gonadotropin (HCG), human luteinizing hormone (HLH), human follicle stimulating hormone (HFSH), and human thyroid stimulating hormone (HTSH) to KS-8 monoclonal antibody was determined using ^{125}I-KS-8 monoclonal antibody (10^5) cpm/well). The bars represent the SEM of triplicate assays

Table 1. Percent cross-reactivity* of monoclonal antibodies produced by clones KS-8 and KS-19 to different related antigens.

Antigen	Monoclonal antibody produced by KS-8 clone	Monoclonal antibody produced by KS-19 clone
Human chorionic gonadotropin	100	100
Alpha-human chorionic gonadotropin	1.2	156
Beta-human chorionic gonadotropin	48.6	3.2
Human luteinizing hormone	0.3	108
Human follicle stimulating hormone	0.2	76
Human thyroid stimulating hormone	0.2	177

* Cross-reactivity was determined as the ratio of human chorionic gonadotropin to the other cross-reactants required for 50% inhibition of monoclonal antibody binding to ^{125}I-human chorionic gonadotropin.

Characterization of monoclonal antibody produced by the KS-19 hybridoma clone The monoclonal antibody produced by the KS-19 hybridoma clone showed the same specific binding capacity as the other α-subunit ligands (Fig. 3). The KS-19 monoclonal antibody showed high affinity to the α-subunit of hCG and low affinity to the β-subunit of hCG (Table 1). Hemagglutination titer of the KS-19 monoclonal antibody was established to be $1{:}10^5$ to $1{:}3 \times 10^5$, which is approximately 20-fold higher than for polyclonal anti-hCG antiserum (Table 2). It was also established that the sensitivity of KS-19 monoclonal antibody for detecting hCG was 0.8 IU/ml of hCG as determined by inhibition of the hemagglutination reaction induced by free hCG (Table 3).

Discussion

For the diagnosis of early-stage pregnancy, radioimmunoassay against anti-hCG antibodies is preferred to the hemagglutination test. Radioimmunoassay requires antibodies with sensitivity against hCG and low cross- reactivity to human LH because hCG shares common α-subunits with the other related glycoprotein hormones. In order to accomplish this, two hybridoma cell lines, KS-8 and KS-19, were developed using the hybridoma technique (Kohler and Milstein, 1975). Two monoclonal antibodies produced by the KS-8 and KS-19

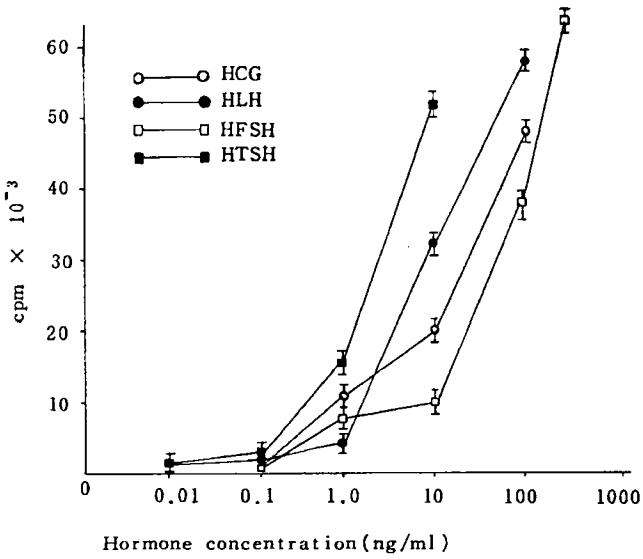

Fig. 3. Characterization of the specificity of KS-19 monoclonal antibody. The binding activity and specificity of the KS-19 monoclonal antibody was determined by the solid phase radioimmunoassay in which plastic microtiter plates were coated with 20 μg purified KS-19 monoclonal antibody. Binding of different concentrations of human chorionic gonadotropin (HCG), human luteinizing hormone (HLH), human follicle stimulating hormone (HFSH), and human thyroid stimulating hormone (HTSH) was determined using ^{125}I-KS-19 monoclonal antibody (10^5 cpm/well). The bars represent the SEM of triplicate assays.

Table 2. Hemagglutination titer of polyclonal and monoclonal anti-human chorionic gonadotropin.*

Antibody	Reciprocal dilution of antibody					
	10^4	2×10^4	10^5	2×10^5	3×10^5	4×10^5
**Polyclonal anti-human chorionic gonadotropin	+	±	−	−	−	−
Monoclonal, clone KS-8	−	−	−	−	−	−
Monoclonal, clone KS-19	+	+	+	+	±	−

* Hemagglutination of sheep red blood cells coated with human chorionic gonadotropin induced by different dilutions of polyclonal and monoclonal anti-human chorionic gonadotropin.
Maximum agglutination (+); intermediate agglutination (±); no agglutination (−).
** A polyclonal antibody obtained by immunization of rabbits with purified human chorionic gonadotropin.

Table 3. Inhibition of hemagglutination induced by human chorionic gonadotropin.*

Antibody	Human chorionic gonadotropin concentration (IU/ml)			
	0.2	0.4	0.6	0.8
**Polyclonal anti-human chorionic gonadotropin	−	−	±	+
Monoclonal, clone KS-19	−	±	+	+

* Hemagglutination of sheep red blood cells coated with human chorionic gonadotropin was induced by polyclonal and KS-19 monoclonal anti-human chorionic gonadotropin. Human chorionic gonadotropin was then added to the reaction mixture and the inhibition of the hemagglutination was monitored. No inhibition (−); partial inhibition (±); complete inhibition (+).
**A polyclonal antibody obtained by immunization of rabbits with purified human chorionic gonadotropin.

cell lines were then characterized with the aid of immunodiffusion, double-antibody radioimmunoassay, and the hemagglutination inhibition test.

KS-8 and KS-19 secreting antibodies belong to the immunoglobulin G_1 subclass.

The monoclonal antibody produced by the KS-8 hybridoma clone recognizes only an immunodeterminant specific to hCG. The cross-reactivity to other pituitary hormones such as human LH, human follicle stimulating hormone, and human thyroid stimulating hormone was found to be less than 0.3%. Monoclonal antibody produced by clone KS-8 thus offers the desired sensitivity, specificity, and affinity required from an antibody for monitoring hCG concentrations in human biochemical fluids by a radioimmunoassay and, therefore, it should be of significant clinical diagnostic interest.

Although the KS-8 monoclonal antibody bound to hCG-coated sheep blood cells, it failed to induce an hemagglutination reaction because it has a high degree of monospecificity and homogeneity. These results were consistent with the study of Milstein *et al.* (1980). Quite contrary to this, a very strong hemagglutination reaction was obtained from the α-hCG-specific KS-19 monoclonal antibody. It was probably due to the heterogeneity of the monoclonal antibody, consistent with the previous studies of Howard *et al.* (1979), and Slaughter *et al.* (1980). It is notable that the hemagglutination titer of the KS-19 monoclonal antibody was significanly higher compared to the titers of polyclonal-heteroanti-hCG antibodies produced by conventional immunological methods.

We expect that this study will contribute to the development of monoclonal antibodies against hCG in the design of clinical assays for confirmation of pregnancy and malignancy. The KS-8 monoclonal antibody originally selected by the radioimmunoassay proved to be a suitable antibody for radioimmunoassay, whereas the KS-19 monoclonal antibody originally selected by the agglutination test proved to be an appropriate antibody for hemagglutination.

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