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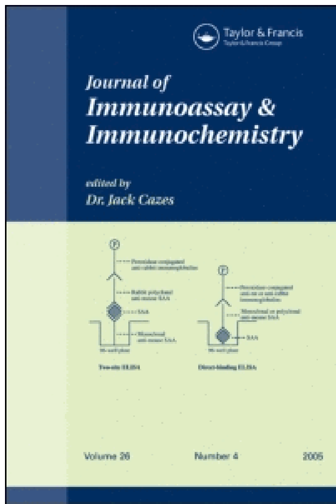

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The Production of High Affinity Monoclonal Antibodies to Human Chorionic Gonadotropin and Their Application to Immunoradiometric Assay

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THE PRODUCTION OF HIGH AFFINITY MONOCLONAL ANTIBODIES TO HUMAN CHORIONIC GONADOTROPIN AND THEIR APPLICATION TO IMMUNORADIOMETRIC ASSAY

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ABSTRACT

Production of monoclonal antibodies against hCG has been studied using hCG as the antigen. This study reports the successful isolation of hybrid clones secreting monoclonal antibodies specific for hCG with an affinity constant higher than $10^{10}M^{-1}$. Of 23 fusions, only 17 fusions have produced positive clones which secrete antibodies giving high levels of binding with ^{125}I-labelled hCG in the supernatant. Finally, 6 different monoclonal antibodies have been isolated; 4 of them, specific for the β-subunit, with a Ka approximately 1.1-4.0 x $10^{11}M^{-1}$ and 2 others, specific for the α-subunit, presenting an affinity of 2.5 x $10^{10}M^{-1}$. When the antibodies specific for the β-subunit are used, specific and highly sensitive radioimmunoassays are obtained after only 3 hrs of incubation. Using iodinated monoclonal antibodies specific for the α-subunit and tubes coated with antibodies against the β-subunit, we have developped sensitive immunoradiometric assays.

Keywords: Radioimmunoassay, immunoradiometric assay, hCG, monoclonal antibodies, glycoprotein hormones.

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INTRODUCTION

Human chorionic gonadotropin (hCG) is a glycoprotein hormone which consists of two distinct subunits (α and β) linked non-covalently. While the α-subunit of hCG is structurally and immunologically very similar to the α-subunit of human luteinizing hormone (hLH), human follicle-stimulating hormone (hFSH) and human thyroid stimulating hormone (hTSH), the β-subunit of hCG is partly different of the β-subunits of these glycoproteins (1). Hence, polyclonal antibodies developped with total hCG as antigen might cross-react strongly with hLH, hFSH and hTSH.

In order to overcome this problem, many investigators attempted to obtain polyclonal antisera by immunizing with the β-subunits of hCG alone (2) or with a segment (residue 109-145) of β-hCG which is a sequence unique to this molecule (3). Unfortunately, the antibodies raised from the β-subunit are not very specific and while a such highly specific requirement is reached when using a segment of β-hCG, it appeared that the low affinity of the antibodies obtained do not allow their use in highly sensitive radioimmunoassay (RIA). Recently, Birken et al. (4) have shown that the preservation of the full carbohydrate content of the segment of β-hCG can play a major role in the generation of antibodies with greater affinity.

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The monoclonal antibody approach may provide the solution of the problem of both the specificity and the sensitivity of the measurement of hCG by RIA. Furthermore, this hybridoma technology can also permit to select two different monoclonal antibodies for the measurement by immunoradiometric assay (IRMA). Several monoclonal antibodies developped against hCG have been reported (5-9) but, except for those reported by Schroder et al. (10), these antibodies show a low affinity constant and cannot be used in a rapid and highly sensitive RIA. As expected, these antibodies give a low sensitivity when used for the analysis by IRMA. Moreover, in accordance with previous observations (9), monoclonal antibodies obtained after immunization with a segment of β-hCG do not lead to the raise of high affinity antibodies.

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Although sorting specific and high affinity antibodies of hCG can be tedious and laborious after injection of the whole molecule, we thought that, with a rapid screening test for high affinity and high avidity antibody, it was possible to rapidly detect those high affinity antibodies and to concentrate our work on these clones. The present study reports the successful isolation of hybrid clones secreting monoclonal antibody specific for hCG, with an affinity higher than $10^{10}M^{-1}$, yet also capable to be used in the measurement of hCG in a sensitive RIA and IRMA.

MATERIALS AND METHODS

Reagents

Highly purified hCG (biopotency, 13400 IU/mg), hLH and hFSH were purchased from Scripp Laboratories, California, U.S.A. The sources of the other reagent are listed as follows: polyethylene glycol 1500 and polyethylene glycol 6000 (PEG 1500 and PEG 6000; BDH Chemicals, Montreal, Canada), tissue culture dishes and flasks (Flow Laboratories, Mississauga, Canada), Dulbecco's modified Iscove's medium (DMIM), foetal calf serum and other tissue culture ingredients (Gibco, Grand Island, N.Y.), chromatography materials (Bio-Rad, Richmond, Ca. and Waters Associates, N.Y.), polystyrene tube (Fisher Scientific, Pittsburgh, PA). The second antibody developed against mice gamma-globulins was prepared in this laboratory by multiple injections in goats of purified gamma-globulin (Miles Laboratories, Elkhart, In.).

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Immunization

Twenty-three 6-8 week old female BALB/c mice were injected subcutaneously with hCG (50 μg/animal) emulsified in 0.2 ml of Freund's complete adjuvent, in three sites. On day 21, two booster injections (100 μg, intravenously in saline solution), were performed at 24-h intervals, followed by cell fusion 3 days later.

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Cell fusion and cloning

Spleen cells (10-15 x 10^7) from immunized mice were removed aseptically and were fused with the mouse myeloma cells NSO/1 (5-7.5 x 10^7) in 50% PEG. After ten days in culture in DMEM medium containing 20% calf serum, hypoxanthine, aminopterin and thymidine, supernatant were tested for anti-hCG antibodies in a screening test using ^{125}I-labeled hCG. The screening test were performed by incubating at 25°C, ^{125}I-labeled hCG diluted in 200 µl of PBS (phosphate-saline buffer, 0.25% bovine serum albumin, pH 7.5) with 15 µl of supernatant diluted three times with the PBS buffer; after 3 hours, 100 µl of second antibody is added, immediately followed by 500 µl of PEG (15% in water). A first selection was made on the basis of the extent of the tracer binding with the antibody in the supernatant. The selected colonies were then transferred into 2 ml wells.

The cells from positive wells were cloned twice in a limiting dilution system and ascite fluid were produced by inoculation into peritoneal cavities of pristane-treated BALB/c mice. The RIA and IRMA were carried out with monoclonal antibodies purified from ascites fluid, using CM Affi-Gel Blue chromatography and HPLC chromatography with a protein pak, DEAE 5PW.

Characterization of antibodies

Monoclonal antibodies were characterized by RIA using a double antibody system. All assays were incubated for 3h at 25°C. Total incubation volume was 300 µl (100 µl of monoclonal antibody diluted in PBS with 1.0% of normal mouse serum, 100 µl of ^{125}I-labelled hCG in PBS and 100 µl of polypeptide standard diluted in PBS) and separation of bound and free ^{125}I-labelled hCG was achieved with the addition of 100 µl of goat anti-mouse gammaglobulin (1:15 final dilution) and 500 µl of PEG 6000 (15% in water). Purified ascites fluid containing large amount of monoclonal antibodies were first titrated for reactivity with ^{125}I-hCG and the dilution were then adjusted to obtain 35% binding of labelled hCG in the absence of unlabeled hCG. Cross-reaction of α and β-subunit of hCG, hLH and hFSH were determined by displacement of labelled hCG. Percent cross-reactivity was calculated from amounts of hormone causing 50% displacement of tracer-labelled hCG. The affinity constants (K_a) were calculated by Scatchard analysis (11).

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Immunoradiometric assay

Coating was achieved by pipetting 0.5 ml carbonate buffer containing 1.0 µg/ml of purified monoclonal antibo-

dies. After 18 hours at 4°, the buffer was aspirated from the tubes which were then incubated for 3 hours at room temperature with 0.5 ml of PBS containing 0.5% human albumin. Tubes were then washed with 0.5 ml distilled water and stored at 4° until used. Monoclonal antibodies 25-245 and 52-46 were iodinated according to the iodo beads method (Pierce Chemicals) and the specific activities of ^{125}I-25-245 and ^{125}I-52-46 were 3 mCi/mg and 11 mCi/mg, respectively.

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Sandwich assays were carried out as follows: various concentrations of hCG or related polypeptide in 100 μl of PBS with 0.25% human serum albumin was pipetted into the tubes followed by the addition of 400 μl of iodinated monoclonal antibody. After mixing, the tubes were incubated at 25°C for 3 hours; the tubes were then decanted, washed twice with water and counted.

RESULTS

Selection of monoclonal antibody

On 23 fusions, only 17 fusions have produced stable and positive clones able to secrete hCG antibodies. Our previous work had demonstrated that, for the screening test, the binding of ^{125}I-hCG with the antibody in the supernatant should be higher than 35% since a lower

binding will result in monoclonal antibody with a low K_a (unpublished observation). Table 1 shows the results of these 23 fusions. A total of 6823 primary hybridoma cultures were obtained from these fusions; however, only 332 media from these cultures gave binding higher than 35%. Forty-two of the 332 original positive cell lines proved to be stable after several weeks in culture. Since sensitive RIA or IRMA need absolutely high affinity antibodies, it can be seen that such antibodies can be rapidly detected and avoid large amount of work with antibodies that are not suitable for hCG measurement. Consistently high levels of binding during cloning was used as a criterium for the selection of the clones subsequently used for antibody production.

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Antibodies characteristics

Of these forty-two clones, six were then chosen on the basis of the rate of antibody secretion, affinity as well as specificity of the monoclonal antibodies as determined with purified antibody from ascitic fluid. The binding capacity of these antibodies has been determined after a 3-hour incubation at room temperature. A 35% binding dilution was then used for the measurement of sensitivity and to perform specificity experiments. Figure 1 illustrates the sensitivity and the specificity of these monoclo-

Table 1

Summary of the production of hybridomas secreting hCG antibodies

Fusion	Number of growing colonies	Number of supernatant with a binding* > 35%	Number of clones giving ascitic fluid
21	315	19	3
22	183	11	1
23	45	2	0
24	345	11	3
25	423	37	4
29	0	0	0
30	589	51	7
31	356	11	0
32	112	7	1
34	214	2	0
35	47	8	2
37	234	3	1
40	345	40	1
41	547	7	1
43	452	13	3
45	456	32	4
46	231	4	1
47	112	1	0
49	231	2	0
50	453	25	4
51	354	22	2
52	348	19	2
54	431	5	2

* Binding to ^{125}I-hCG after a three-hours incubation.

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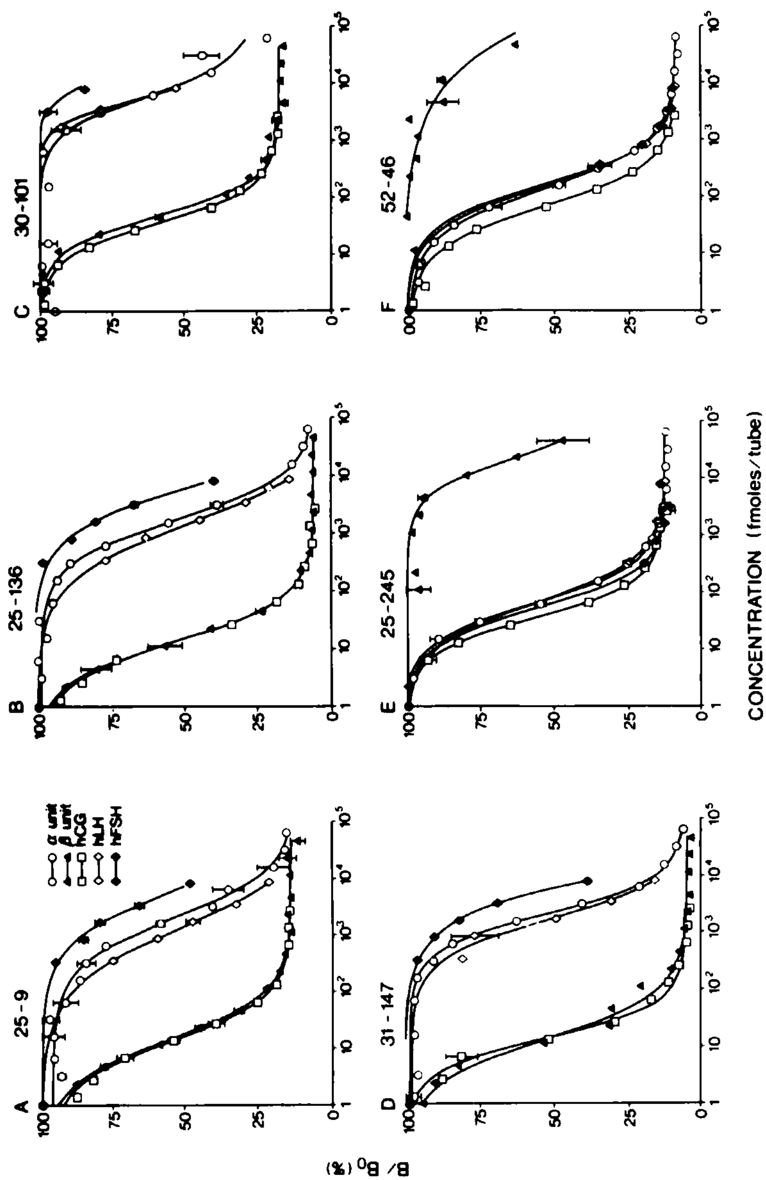

Fig. 1. Calibration curve of monoclonal antibodies 25-9, 25-136, 30-101, 31-147, 25-245 and 52-46 with hCG, α-hCG, β-hCG, hLH and hFSH.

nal antibodies as determined by competition with hCG, α-hCG, β-hCG, hLH and hFSH. It can be seen that antibodies 25-9, 25-136, 30-101 and 31-147 cannot only bind hCG and the β-subunit, but also showed less than 0.8% cross-reactivity with the α-hCG, hLH and hFSH. However, two other monoclonal antibodies (25-245 and 52-46) which can bind to ^{125}I-hCG also show a cross-reaction with the α-subunit of hCG (54 and 49%, respectively) and, as expected, exhibit high affinity for hLH as well as hFSH while they cross-react less than 0.2% with the β-subunit of hCG. Further examination of the specificity of these six antibodies also indicate that none of these monoclonal antibodies reacted with the other pituitary hormones (data not shown). Moreover, we obtained a small percentage (3/42) of monoclonal antibodies against the α-subunit of the glycoprotein in response to immunization with native hCG.

Table 2 shows the affinity constant of these six monoclonal antibodies; it can be seen that there are two groups of K_a. The antibodies 25-9, 25-136, 30-101 and 31-147 which are specific to the β-subunit show a K_a of 1.1-4.0 x 10^{11} while antibodies 52-46 and 25-245 which bind the α-subunit show a lower K_a (2.2 x 10^{10} and 5.6 x $10^{10}M^{-1}$, respectively).

IRMA standard curve

Figure 2 illustrates six IRMA standard curves generated by the incubation of iodinated monoclonal antibodies

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Table 2

Characteristics of monoclonal antibodies produced against hCG

Hybridoma clone	% cross-reaction				Affinity constant (M^{-1})
	α-hCG	β-hCG	hLH	hFSH	
25-9	0.7	97	0.8	0.3	1.4×10^{11}
25-136	0.7	98	0.8	0.1	1.1×10^{11}
30-101	0.6	72	0.8	0.7	2.0×10^{11}
31-147	0.7	104	0.8	<0.1	4.0×10^{11}
25-245	54	0.2	51	65	5.6×10^{10}
52-46	49	<0.1	40	45	2.2×10^{10}

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25-245 and 52-46 which recognized specifically the α-subunit of hCG and with monoclonal antibody (25-9, 30-101 or 31-147) which are specific for the β-subunit, coated on polystyrene tubes. Unfortunately, it was not possible to coat on these tubes monoclonal antibody 25-136 and several attempts with different buffers and various pH remained unsuccessful.

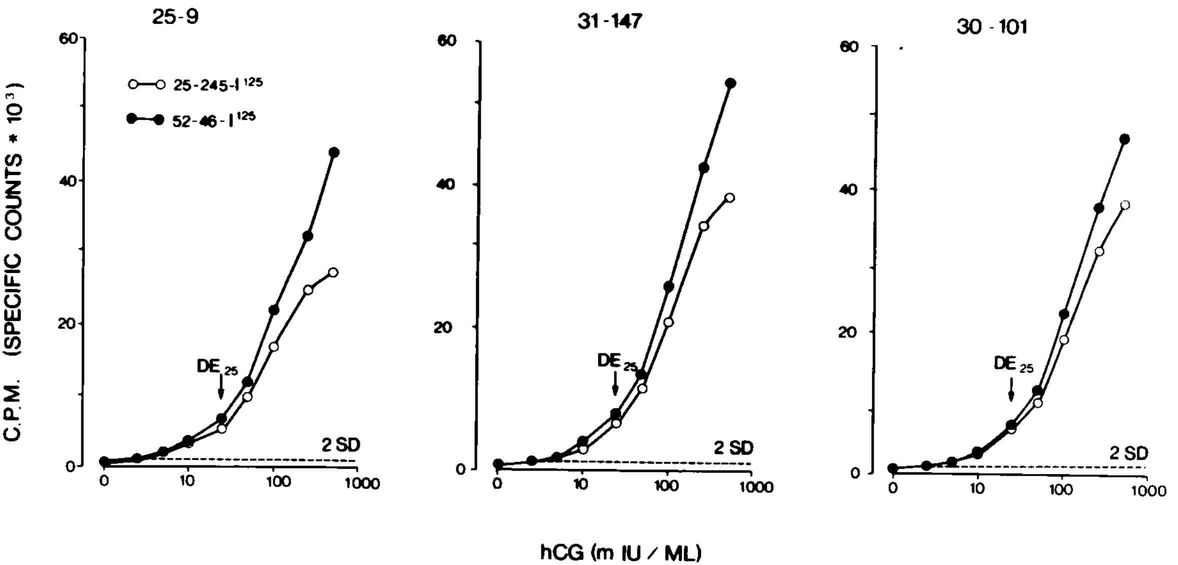

Fig. 2. Immunoradiometric assay with the combination of two monoclonals antibodies (25-9 and 25-245; 25-9 and 52-46; 30-101 and 25-245; 30-101 and 52-46; 31-147 and 25-245; 31-147 and 52-46). Assay is simultaneous sandwich and is performed at room temperature for 3 hrs.

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The non-specific binding (NSB) in the assay was 0.1-0.3% of the total counts added (180,000-200,000 cpm) and the least detectable dose of hCG has been calculated as the hCG concentration resulting in an increase in cpm bound that was 2 SD higher than the mean (n = 6) of NSB (Fig. 2). We have also indicated the specific count obtained at the dose of 25 mIU/ml (DE_{25}; fig. 2) which is generally, in clinical assay kit, the cutt-off dose of detectable hCG in the beginning of pregnancy. The standard curve for hCG was established between 1 mIU/ml and 500 mIU/ml.

It can be seen that, after a three-hour period of incubation, sensitive IRMA is obtained in all combinations using these high affinity monoclonal antibodies (Fig. 2). It appears that no interference between antibodies seems to occur when such a combination of monoclonal antibodies specific for the α-subunit and the β-subunit are used. These systems can accurately and rapidly measure 1 mIU/ml and we have observed a specific binding of 7-10 fold of background counts at the dose of 25 mIU/ml (Fig. 2). The determination of the cross-reactivity in the IRMA indicate that the levels of interaction with α-hCG, hLH and hFSH are slightly higher (1-7%) to those observed when using the coated antibody alone in RIA (data not shown).

DISCUSSION

In the present study, we have clearly demonstrated that stable clones which secrete monoclonal antibodies to

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hCG with high specificity and high affinity constant are produced after injection of the immunogen hCG. We have also shown that these antibodies can be used to quantify hCG in highly sensitive RIA and IRMA. In addition, our data indicate that, as expected, the immunization with the whole hCG molecule induces the formation of antibodies which recognize specifically the α or the β subunit; however, we have detected much more monoclonal antibodies with high affinity against the β-subunit than those of α-subunit and, furthermore, the monoclonal antibodies specific for the β-subunit possess an higher affinity for hCG. To our knowledge, it is the first time that this observation on the high immunological property of the β-subunit of hCG compared to the α-subunit is reported.

The presence of a specific epitope of hCG in the β-subunit section which is recognized by antibodies with high affinity has been already reported (2, 10, 12). More interestingly, it has been shown that this epitope is not present in the 30-amino acid segment of the β-subunit that is absolutely unique to β-hCG (2, 4). The type of polyclonal antibody that have been generated by this epitope has been called "SB-6" and shows minor cross-reactivity with hLH (4).

Recent data reported by Birken et al. (4) indicated that other epitopes might also exist in the β-COOH-terminal structure of hCG and gives antibodies with high affinity.

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However, the immunogen has to contain the carbohydrate chain in order to develop high affinity antibodies (4). Previous study with polyclonal and monoclonal antibodies have clearly shown that the use of synthetic peptide without the carbohydrate residue lead to the formation of antibodies with low affinity (5, 6). Our present data are in agreement with observations on the presence of a specific and high affinity antibody binding site in the β-subunit of hCG; however, further work has to be performed in order to identify the site of this epitope.

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While a large number of screening tests has been performed, our data indicated that 95% of our positive growing hybridoma clones can be rapidly removed and the subsequent work is performed with clones that secrete high affinity antibodies. On these 5% remaining, almost 90% were not viable in the second culture and it was then easier to focus our work on the positive hybridoma. Of the 42 clones secreting an antibody which gives high levels of binding with ^{125}I-hCG, we found that all these clones secrete an antibody with an affinity higher than 1.5 x $10^9 M^{-1}$ (data not shown). However, 36 antibodies isolated were not specific or showed an affinity constant between 10^9 and $10^{10} M^{-1}$ for hCG and thus cannot be used in sensitive IRMA and RIA.

While some IRMA and Elisa have been recently described (7, 8, 12), the results obtained in the present study

show that dramatic sensitivity can be reached with our monoclonal antibodies which have an affinity higher than $10^{11}M^{-1}$. Hence, it is thus possible to measure, in a three-hours incubation, as low as 5 mIU/ml of hCG. Moreover, this sensitivity allow to develop a sensitive "simultaneous" analysis by IRMA which is becoming more widely accepted in clinical laboratories. The next step will be to demonstrate that this monoclonal antibody IRMA described here has the specificity and the sensitivity to measure serum hCG in the clinically important range at the beginning of pregnancy.

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