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Production of antibodies specific to human chorionic gonadotropin in mice immunized against its chemical analogs†

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Abstract. Mice immunized against DS_5-hCG-β and DS_6-hCG-β, chemical analogs of β-subunit of human choriogonadotropin (hCG-β) in which 5 and 6 disulphide bonds respectively were reduced and alkylated, were found to produce antibodies specific to hCG without significant crossreactivity with human lutropin (hLH) as tested in a radioimmunoassay. In contrast, mice immunized against the native hCG-β subunit produced hLH crossreacting antibodies. While the anti-DS_5, DS_6-hCG-β serum was capable of selectively blocking the binding of [^{125}I]-hCG to rat testicular LH/hCG receptors, it failed to inhibit the binding of [^{125}I]-hLH to the same receptors. The radioimmunoassay for hCG using the mouse anti-DS_5, DS_6-hCG-β serum was not as sensitive as that employing rabbit anti-DS_5, DS_6-hCG-β serum. The minimal detection limit was 5 ng/ml for the mouse antibody as compared to 1 ng/ml for the rabbit antibody.

Keywords. hcG Specific antibody; disulphide modified hCG-β-subunit; immuno-contraceptive.

Introduction

Although antibodies to human choriogonadotropin β-subunit (hCG-β) can discriminate between hCG and human lutropin (hLH), they lack absolute specificity. The degree of hLH crossreactivity of the antibody varies with the preparation of hCG-β, ranging from 10 to 30%. Nevertheless, antibody to hCG-β is considerably more specific than antibody to hCG and this specificity is most likely due to the differences in the physicochemical characteristics of hLH and hCG-β (Bhal, 1980). hCG-β not only has an additional 30-amino acid residue sequence at its carboxy-terminus but also has certain other domains in the polypeptide chain with variable sequences. Furthermore hCG-β undergoes a

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Abbreviations used: hCG, human choriogondotropin;hCG-α and hCG-β, α and β subunits of hCG; hLH, human lutropin; hFSH, human follitropin; hTSH, human thyrotropin; hPRL, human prolactin; DS_5hCG-β-hem and DS_6hCG-β-hem, reduced and S-alkylated hCG-β in which 5 and 6 disulphide bonds were modified respectively and then coupled with hemocynanin; RIA, radioimmunoassay; CMC, carboxymethyl cysteine; TC buffer, 0.05 M Tris-HCl buffer pH 7.2 containing 1 mM $CaCl_2$.

conformational change when it is dissociated from hCG-α (Hilgenfeldt *et al*., 1974). The antigenic determinants being conformational in nature, this change in the conformation results in the loss of hLH crossreactivity of the sites. We have attempted to modify intact hCG-β in order to enhance its specificity as an antigen rather than use the unique carboxyterminal peptide which is a weak antigen (Matsuura *et al*., 1979). Earlier work from our laboratory has demonstrated that progressive reduction and alkylation of disulphide linkages in hCG-β results in loss of antigenic determinants common to hCG and hLH (Bahl *et al*., 1976). Among the various derivatives, reduced and S-alkylated hCG-β in which 5-disulphide bonds were modified (DS_5-hCG-β) retained only determinants unique to hCG with complete loss of those crossreacting with hLH antibodies (Ghai *et al*., 1980). Immunization of rabbits with DS_5-hCG-β prepared according to the methods standardized in our laboratory yielded antibodies specific to hCG by immunological and biological criteria (Pandian *et al*., 1980). With the idea that this chemical derivative of hCG-β would be useful as a means of immunological control of fertility in humans, studies were initiated to test the immunogenic specificity of the antigen in other animals. We report here that a sequential injection of DS_5-hCG-β and DS_6-hCG-β, a fully reduced and alkylated derivative of hCG-β, into mice resulted in antibodies specific to hCG. As a control, another group of mice was immunized against native hCG-β. This group of mice produced antibodies to hCG which were crossreactive with hLH to a significant extent.

Materials and methods

Sephadex-G-25 (coarse), G-100, Sepharose-4B and diethylaminoethyl (DEAE) Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey, USA. Freund's complete adjuvant was a product of Gibco, Grand Island, New York, USA. Carrier free $Na^{125}I$ was purchased from Amersham Corp., Arlington, Illinois, USA. Bovine-γ-globulin, bovine serum albumin and glutaraldehyde (25% solution) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. All other chemicals were of certified Analytical grade. BALB/C mice were used in this study.

Preparation of DS_5-hCG-β and DS_6-hCG-β

The β-subunit of hCG was prepared by dissociation of hCG in 8M urea and chromatography over DEAE-Sephadex-A 50 (Swaminathan and Bahl, 1975). To remove the last traces of hCG, the β-subunitwas treated with an immunoadsorbent prepared by coupling antibodies to hCG-α to CNBr-activated Sepharose (Pandian *et al*., 1980). After determining the binding capacity of the immunoadsorbent hCG-β was treated with a calculated amount of the immunoadsorbent. Details of the procedure are given elsewhere (Pandian *et al*., 1980). The anti hCG-α immunoadsorbent treated hCG-β was then partially or completely reduced and alkylated to prepare DS_5-hCG-β and DS_6-hCG-β respectively (Ghal *et al*., 1980). Amino acid analysis for carboxymethylcysteine residues was later carried out to confirm the degree of reduction. The hLH cross-reactivity of the preparation was monitored by radioimmunoassay of DS_5-hCG-β in a [^{125}I]-hLH-anti-hLH radio immunoassay (RIA) system. In order to ensure the complete removal of hLH

crossreactivity of the antigen, it was treated with a calculated amount of an immunoadsorbent, prepared by coupling antibodies to hLH to CNBr-activated Sepharose. The efficacy of the treatment was monitored by a homologous hLH RIA. This material was then conjugated to Keyhole limpet hemocyanin in a 1:1 ratio (w/w) using glutaraldehyde as the bifunctional cross-linking reagent (Pandian *et al*., 1980). The resulting conjugate, DS_5-hCG-β -hem and DS_6-hCG-β were used to immunize mice.

Immunization of mice and collection of blood samples

Mice were immunized by intraperitoneal injection. A water in oil emulsion was prepared by mixing equal volumes of a saline solution of the immunogen [hCG-β or DS_5-hCG-β coupled with hemocyanin (DS_5-hCG-β-hem) or DS_6-hCG-β] and Freund's complete adjuvant. Mice received two injections of DS_5-hCG-β-hem (250 µg/mouse) one month apart. Twenty day after the second injection they received DS_6-hCG-β (100 µg/mouse). A second group of mice was immunized similarly by these injections of native hCG-β-subunit (100 µg/mouse). Blood samples were collected by occular puncture. Serum was later separated and frozen with 0.01% sodium azide as a preservative.

Titer of antisera

Serum (200 µl) at various dilutions (1:10 to 1:10,000) was incubated with [^{125}I]-hCG for 1 hat 37°C followed by the addition of 10 µl of 10% ammonium acetate in ethanol. The tubes were kept for 1 h at 4°C, then centrifuged, supernatants decanted, residual liquid removed by wiping with filter paper strips and finally the pellets were counted for antibody bound readioactivity. The dilution of the antiserum binding 20% of the [^{125}I]-hCG (40-50 µCi/µg) was chosen for radioimmunoassay.

Radioiodination and radioimmunoassay

The radioiodination of highly purified hCG for RIA was performed essentially by the chloramine-T procedure of Greenwood *et al*. (1963) with minor modifications.

The radioimmunoassay was performed essentially as per the procedure of Bellisario and Bahl (1975) . The results were expressed as $B/B_0 \times 100$ and plotted against log dose of hCG standards. B_0 represents cpm radioactivity bound in the absence of any competing antigen and B represents cpm radioactivity bound in the presence of various levels of competing standard hCG or unknown sample. The radioimmunoassay data were also analyzed by a computer, Wang Model 2200, using program RIADS-2200, based on the method developed by Rodbard and Lewald (1970).

Radioimmuno-receptor assay

Rat testicular homogenate was used as a source of hCG/hLH receptors. Testes were removed from a healthy adult rat and placed on ice. After decapsulating the pair of testes was homogenized in 20 ml of 0.05 M Tris-HCl buffer pH 7.2 containing 1mM $CaCl_2$ (TCbuffer) in a motorized Potter-Elvehjem tissue grinder

(no more than 6 strokes). The homogenate was centrifuged at 1,000 *g* for 15 min in a Sorvall-RC 2B refrigerated centrifuge. The pellet was resuspended in 40 ml of TC buffer containing 0.2% BSA. Antisera (200 µl) serially diluted or standards were incubated with 0.5 ml aliquots of the testicular homogenate and 100,000 cpm of [^{125}I]-hCG or [^{125}I]-hLH for 1 h at 37°C in a Dubnoff metabolic shaker. At the end of the incubation, the tubes were centrifuged at 4°C at 2000 *g* for 15 min, the supernatants decanted, the pellets washed with 2.5 ml of chilled TC buffer by resuspension and centrifuged at 2000 *g* for 15 min. The supernatants were decanted, and the liquid adhering to the walls wiped with filter paper strips. The pellets were counted for receptor bound radioactivity. Results were expressed as $B/B_0 \times 100$ *vs* log dose of Standard hormone or dilution of antiserum. B_0 represents radioactivity (cpm) bound to receptors in the absence of competing standard hormone or antiserum and B represents radioactivity bound to the receptors in the presence of standard hormone or antiserum. Nonspecific binding was determined by obtaining cpm bound in the presence of excess of the cold hormone (hCG or hLH).

Results and discussion

The antigen

The hCG-β subunit prepared by dissociation of highly purified hCG with urea had 0.5% contamination as found by the radioreceptor assay. When treated with an immunoadsorbent of anti hCG-α antibodies, the contamination (hCG) was completely removed (Pandian *et al*., 1980). This hCG-β was then reduced partially under controlled conditions and alkylated to yield DS_5-hCG-β On analysis, a value of 10.4 was obtained for carboxymethylcysteine residues pointing to the reduction on an average of 5.2 disulphide bridges (Ghai *et al*., 1980). The material was tested in [^{125}I]-hLH-and-hLH RIA system to monitor the presence of hLH-cross reacting material. This amounted approximately to 0.2-0.3% (figure 1) of hLH activity. However, after treatment with an

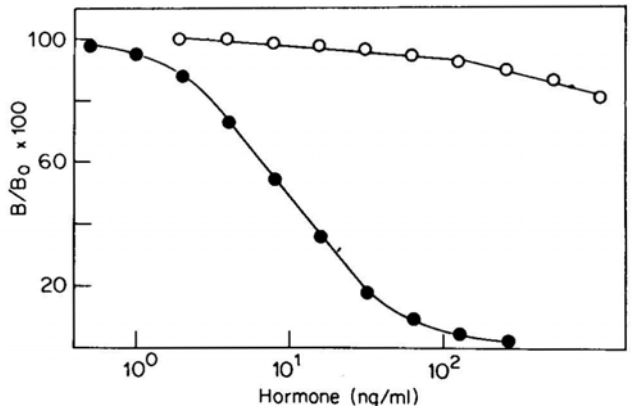

Figure 1. Radioimmunoassay of DS_5-hCG-β in the ^{125}I-hLH-anti-hLH system. Human LH (●)and DS_5-hCG-(O) were separately used as standards in an RIA using ^{125}I-hLH and anti-hLH serum (1:30,000 dilution of antiserum). For details of the assay, see the text. Results are expressed as $B/B_0 \times 100$ vs log dose of the competing antigen. The value of B_0 ranged from 9,000-10,000 cpm corresponding to 27% to 30% specific binding.

immunoadsorbent of anti-hLH-Sepharose, the crossreactivity was reduced to negligible level (0.01 % hLH activity). DS_5-hCG-β conjugated to Keyhole limpet hemocyanin was then dialyzed and lyophilized to yield DS_5-hCG-β-hem. DS_6-hCG-β was prepared by complete reduction and alkylation of hCG-β. Amino acid analysis gave an average value of 11.96 for the number of CMC residues indicating that 5.98 disulphide bridges had been reduced and alkylated (Ghai *et al*., 1980).

The antisera

Mice were immunized against native hCG-β or DS_5-hCG-β-hem as described under Methods. The serum levels of antibody after the second injection are shown in Figure 2. The anti-DS_5-hCG-β-hem serum showed very minimal

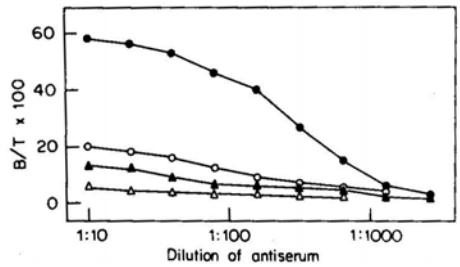

Figure 2. Titer and crossreactivity of anti-hCG-β serum and anti DS_5-hCG-β serum from mice immunized against the respective antigens. Data from a representative mouse is given here for each antigen. For details of procedure see the text. Results are expressed as B/T × 100 *vs* log dilution of antiserum where B represents cpm bound to the antibody and T is the total cpm added to the incubation.(●) Binding of ^{125}I-hCG to anti-hCG-β serum; (▲) Binding of ^{125}I-hLH to anti-hCG-β serum; (O) Binding of ^{125}I-hCG to anti-DS_5-hCG-β serum; (Δ) Binding of ^{125}I-hLH to anti-DS_5-hCG-β serum.

binding to [^{125}I]-hLH. However, after receiving the third injection (with DS_6-hCG-β), the antibody level against hCG was elevated with negligible binding to [^{125}I]-hLH (figure 3). In comparison, the serum from mice immunized against

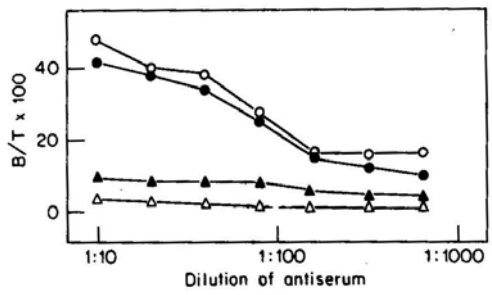

Figure 3. Titer and crossreactivity of anti-hCG-β serum and anti-DS_5, DS_6-hCG-β serum from mice immunized against the respective antigens. Data from one representative mouse is given here for each antigen. For details of procedure see the text. Results are expressed as B/T × 100 dilution of the antiserum. For explanaton, see the legend to figure 2.

native hCG-β, bound [^{125}I]-hLH to an appreciable extent. When tested in an RIA using 1:100 dilution, hLH did not interfere to any appreciable degree when anti-DS_5-hCG-β serum was used (figure 4). In the case of anti-hCG-β serum, however, hLH inhibited the binding of [^{125}I]-hCG to a significant degree (from 40 ng/ml and

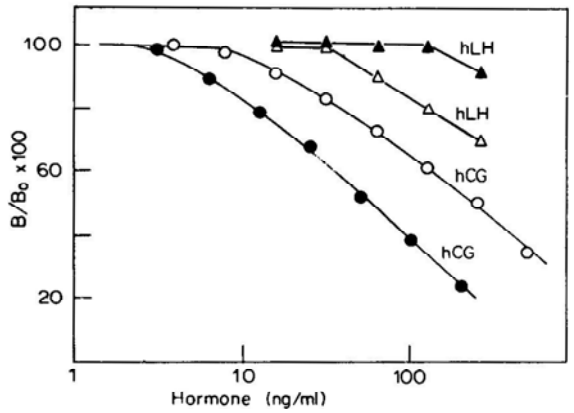

Figure 4. Radioimmunoassay of hCG and hLH using 1:100 dilution of mouse anti-hCG-β (O) and anti-DS_5-, DS_6-hCG-β sera (●). For details of the assay see the text. Results are expressed as $B/B_0^{\times 100}$ *vs* log dose of the competing antigen where B represents cpm bound to the antiserum in the presence of graded doses of the competing antigen and B_0 represents cpm bound to the antiserum in the absence of any competing antigen. The value for B_0 was 8000 cpm representing 20% specific binding.

higher). The sensitivity (minimal detection limit) of the assay using mouse anti-DS_5, DS_6-hCG-β-hem serum was 5 ng/ml. This, however, was lower than that of the rabbit antibody based RIA which could measure 1 ng/ml with confidence (K. Muralidhar, G. Chaudhuri, J. Lippes and O. P. Bahl–unpublished data). This confirms and extends our earlier report that the rabbit antibody to DS_5, DS_6-hCG-

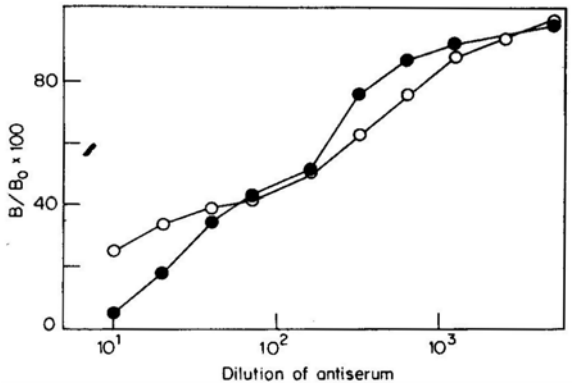

Figure 5. Radioreceptor assay of hCG in the presence of antibodies to DS_5, DS_6-hCG-β from rabbits (O) and mice (●). Rat testicular homogenate (0.5 ml) was incubated with [^{125}I]-hCG and serial dilutions of rabbit or mouse anti-DS_5, DS_6-hCG-β serum. At the end of the incubation, the receptor bound radioactivity was determined. For details see the text. Resuls are expressed as $B/B_0 \times 100$ where B and B_0 represent cpm bound in the presence or absence of various dilutions of either rabbit or mouse antibody. The value for B_0 was 10,000 cpm representing 13% specific binding to receptors.

β was specific to hCG with little or no crossreaction with hLH (Pandian *et al*., 1980). In order to ascertain whether the mouse anti-DS_5, DS_6-hCG-β serum was capable of selective neutralization of biological activity of hCG, the radio-receptor assay was carried out. As shown in figures 5 and 6 the mouse antibody was capable

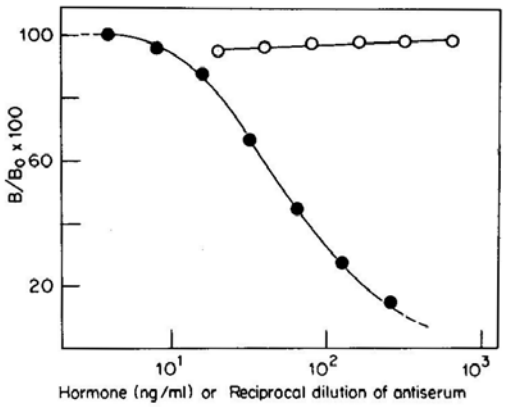

Figure 6. Radioreceptor assay of [^{125}I]-hLH in the presence of antibodies to DS_5, DS_6-hCG-β from mice. Rat testicular homogenate (0.5 ml) was incubated with [^{125}I]-hLH and serial dilutions of mouse anti-DS_5, DS_6-hCG-β serum (O) or standard hLH (●) in amounts indicated in the figure. At the end of the incubation, the receptor bound radioactivity was determined. For details see the text. Results are expressed as $B/B_o \times 100$ where B_o represents cpm bound in the absence of any competing LH or antibody and B represents cpm bound in the presence of various dilutions of the antibody or standard hLH. The value for B_o was 9000 cpm representing 10% specific binding to receptors.

of blocking the binding of [^{125}I]-hCG to testicular receptors. For comparison, the effect of rabbit antibody on the binding of [^{125}I]-hCG to receptors is also depicted in figure 5. In contrast, the binding of [^{125}I]-hLH to testicular receptors was unaffected by the presence of mouse anti-DS_5, DS_6-hCG-β serum (figure 6). This again demonstrates and confirms the biological specificity of the antibody.

A number of placental antigens have been proposed as immuno-contraceptive agents (Bhal and Muralidhar, 1980). Among these only hCG has been investigated in great detail. Active immunization studies in subhuman primates involving hCG-β have demonstrated the possibility of interrupting pregnancy with this immunogen (Hearn, 1979). These studies have, at the same time, indicated the existence of hLH crossreactivity in the anti-hCG-β antibodies. The degree of crossreactivity with hLH, however, varied from animal to animal. As the ideal placental immuno-contraceptive agent is envisaged as one eliciting antibodies specific to itself without cross reactivity with any non-placental tissue antigen including pituitary LH, there is a need to improve the specificity of hCG-β antibodies. Although the unique carboxy terminal peptide of hCG-β produces antibodies to hCG, some of which are capable of neutralizing the biological activity of hCG-β an action through which it is hoped to terminate pregnancy, they are not specific. Hence, an alternative approach, presented here and elsewhere (Pandian *et al*., 1980) to use chemical derivatives of hCG-β was investigated. These studies revealed that

among the derivatives of hCG-β, DS_5-hCG-β and DS_6-hCG-β were least crossreactive with anti-hLH sera (Ghai *et al.,* 1980). It has been shown here that mice generate antibodies specific to hCG when they are immunized against DS_5-hCG-and DS_6-hCG-β Although in a direct binding assay, the antibodies displayed binding to [^{125}I]-hLH, the binding was of low affinity as compared with the binding to [^{125}I]-hCG. This was evident from the fact that in a competitive test system such as RIA or RRA, the antibody did not show any binding to hLH. In contrast, the antibodies to hCG-β bound [^{125}I]-hLH in a direct binding assay and this binding apparently was of considerable affinity as even in an RIA the antibody binding to [^{125}I]-hCG was inhibited by the unlabeled hLH.

The immunological and biological specificity of the anti DS_5, DS_6-hCG-β sera in both rabbits (Pandian *et al*., 1980) and mice points to the possible use of DS_5, DS_6-hCG-β as an immunocontraceptive agent. The lymphocytes from these mice immunized against DS_5-hCG-β and DS_6-hCG-β have been used to develop monoclonal antibodies to hCG without any cross reaction with hLH (Muralidhar *et al*., 1981). The biological effects of active immunization with DS_5-hCG-β and DS_6-hCG-β are presently being investigated in a number of laboratory animals including subhuman primates.

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