Identification of a heterodimer-specific epitope present in human chorionic gonadotrophin (hCG) using a monoclonal antibody that can distinguish between hCG and human LH

R A Gadkari, S Roy, N Rekha[1], N Srinivasan[1] and **R R Dighe**

Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India

[1]Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

(Requests for offprints should be addressed to R R Dighe; Email: rdighe@mrdg.iisc.ernet.in)

Abstract

Human chorionic gonadotrophin (hCG) is secreted during early pregnancy and is required for implantation and maintenance of the pregnancy. Active or passive immunoneutralization of hCG results in termination of pregnancy and this forms the basis of the hCG-based female contraceptive vaccine. However, the β subunit of hCG possesses 85% sequence homology with the first 114 amino acids of the β subunit of pituitary human LH (hLH), which is required for ovulation and maintenance of the corpus luteum function during the menstrual cycle. Immunization against hCG or its β subunit leads to generation of antibodies that can neutralize hLH due to many shared epitopes and hence may cause abnormal menstrual cycles. Therefore, it is essential to identify epitopes that are different in the two hormones. In the present study, we report a monoclonal antibody (MAb) specific for hCG that shows no binding to the isolated subunits. Interestingly, the MAb also does not bind hLH at all. The epitope mapping analysis revealed that this antibody recognizes a unique discontinuous epitope present only in the heterodimeric hCG and is distinct from the unique C-terminal extension of hCGβ that is absent in hLHβ. The MAb, either as IgG or its recombinant single-chain variable region fragment, inhibited the response to hCG, but not to hLH. Thus, the epitope recognized by this MAb is an ideal candidate antigen for immunocontraception.

Journal of Molecular Endocrinology (2005) **34,** 879–887

Introduction

Human chorionic gonadotrophin (hCG) is secreted by the trophoblastic cells of the placenta during early pregnancy (Fox 1970). hCG plays an important role in the rescue of the corpus luteum and maintenance of pregnancy (Strauss *et al.* 1995). Active or passive immunoneutralization of hCG prevents implantation of the embryo, which is the basis of the female contraceptive vaccine (Talwar *et al.* 1994, Dirnhofer & Berger 1995, Stevens 1996, Delves 2004). One such vaccine has completed phase II efficacy trials in women (Talwar *et al.* 1994). The immunogen for this vaccine consists of a heterospecies dimer (HSD) of the β subunit of hCG (hCGβ) associated non-covalently with the α subunit of ovine luteinizing hormone (oLHα) and conjugated to either tetanus or diphtheria toxoid as carrier (Talwar *et al.* 1994). The results of the phase II clinical trials with this HSD vaccine revealed that sexually active women with normal ovulatory cycles did not become pregnant as long as the circulating antibodies were above 50 ng/ml, with no effect on the luteal phase length, and exhibited menstrual regularity, demonstrating the contraceptive efficacy of the vaccine.

However, hCGβ possesses 85% sequence homology with the first 114 amino acids of the β subunit of human LH (hLH), which is not a pregnancy-specific hormone but plays a critical role in ovulation and maintenance of the corpus luteum function in the luteal phase of the female reproductive cycle. Immunization against hCG can thus lead to immunological cross-reaction with hLH due to many shared epitopes between the two hormones, and although the phase II trial of the HSD vaccine did not show any effect on hLH function, prolonged immunization may eventually lead to defects in ovulation and luteal phase defects (Dirnhofer & Berger 1994). In order to circumvent possible cross-reactivity with hLH, attempts have been made to use as the immunogen the 30 amino acid C-terminal peptide (CTP) that is unique to hCGβ but absent in hLHβ. Antibodies against this peptide are highly specific for hCG, but cannot neutralize the biological activity of the hormone (Louvet *et al.* 1974). In addition, the CTP is a poor immunogen and generates antibodies with low affinity and lower bioneutralization capacity (Mitchison 1990). Hence, it is essential to identify the epitopes in hCG molecule, other than the CTP region, that are absent in hLH so that these epitopes can serve as

potential targets for eliciting highly specific hCG bioneutralizing antibodies in humans.

In the present study, we report a monoclonal antibody (MAb) designated as E12 that displays very-high-affinity binding to hCG specifically and does not cross-react with hLH, as well as the subunits of hCG. Using different strategies, a conformation-specific epitope within the hCG molecule distinct from CTP has been identified.

Materials and methods

Chemicals and biological materials

Hormones

The hormones and their subunits – hCG (CR127), hLH, human follicle-stimulating hormone (hFSH), human thyrotrophin (hTSH), hCGα, hCGβ (CR123) – used in this study were obtained from The National Hormone and Pituitary Program, UCLA, USA.

Biochemicals and radiochemicals

^{125}I NaI and [1,2,6,7,16,17-^{3}H]-testosterone were purchased from Perkin-Elmer Life Sciences, Boston, MA, USA. All the restriction enzymes and modifying enzymes required were purchased from Boehringer Mannheim, or from MBI Fermentas (Hanover, MD, USA). Phenyl Sepharose, HiTrap Protein G-Sepharose columns, HiTrap chelating columns used for purification of IgG and recombinant single-chain variable region fragment (ScFv) were purchased from Amersham Pharmacia Biotech. All other reagents were obtained from Sigma Chemical Company.

Production, characterization and purification of MAbs

MAbs against hCG and *Pichia*-expressed recombinant hCG used in the present study were raised and characterized as described previously (Dighe *et al.* 1990*a*). The ability of these antibodies to bind all human glycoprotein hormones or their subunits was demonstrated using ^{125}I-labelled probes or ELISAs. Ascites were produced in Balb/c mice and were used for purification of IgG by Protein G-Sepharose chromatography. All animal experiments were carried out under expert supervision after obtaining appropriate clearances from the Institutional Ethical Committee that oversees the usage of animals in the institute.

RIA

To test binding of the antibody to various antigens, ^{125}I-hCG/hCGα/hCGβ/hFSH/hTSH/hLH, were incubated overnight with the desired amount of antibody at room temperature. The antigen–antibody complexes were precipitated by the addition of anti-rabbit IgG raised in goat and polyethylene glycol 6000 as described earlier (Dighe *et al.* 1990*a*, Sen Gupta & Dighe 1999), centrifuged at 5000 **g**, the supernatant discarded and the radioactivity in the pellet counted using a Perkin-Elmer autogamma counter.

ELISA

hCG, hLH and single-chain derivative of hCG (hCGβ) and its mutants (50 ng/well) were coated on ELISA plates followed by 1% BSA solution in PBS as blocking buffer. The MAbs (purified IgG or ScFv) were then added to the wells and incubated at 37 °C for at least 1 h and binding was determined as described earlier (Gadkari *et al.* 2003).

Cloning of single-chain antibody (ScFv)

The single-chain antibody (ScFv) (Huston *et al.* 1988) was generated from the hybridoma cells producing MAb E12 using the kit purchased from Pharmacia Biotech employing the protocol provided by the manufacturer. The antigen binding ability of the ScFv was determined by ELISA and its ability to inhibit hCG–receptor interaction was determined by a radioreceptor assay (RRA) as described in the case of the parent antibody. The cDNA encoding the ScFv was cloned into *Pichia* expression vector, pGAPZαA (Invitrogen) and fermentation with the clone secreting the ScFv was carried out as described for hCG (Gadkari *et al.* 2003). ScFv secreted into the medium was purified using a combination of hydrophobic interaction chromatography and Ni^{2+}-affinity chromatography as described in the *Current Protocols in Protein Science, Volume I Supplement 1 CPPS Unit 9·4.*

RRA

The effect of antibodies on hCG–receptor interaction was determined by incubating the antibodies with ^{125}I-hCG for 1 h at 37 °C followed by addition of particulate rat LH receptor and continuing the incubation for additional 1 h. ^{125}I-hCG bound to the receptor was pelleted by centrifugation at 5000 **g** and counted in a gamma counter (Sen Gupta & Dighe 1999). The total binding was determined by incubating the receptor with either buffer or normal rabbit serum, while the non-specific binding was determined by incubating ^{125}I-hCG with the receptor in the presence of 400 ng/ml unlabelled hCG and was subtracted from the total binding to obtain specific binding.

In vitro *bioassay*

The *in vitro* bioassay was performed using mouse testis cell preparation as the source of the Leydig cells as

described earlier (Dighe & Moudgal 1983). Briefly, the Leydig cells were obtained by mechanical dispersion of adult mouse testes and suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 50 mM Hepes and 0·1% BSA. hCG was preincubated with the desired antibody diluted in DMEM, Hepes and BSA for 1 h, after which the cell preparation was added to it, and the incubation was continued for another 4 h. At the end of this incubation period, the medium was separated from the cells by centrifugation and testosterone secreted into the medium was estimated by RIA as described earlier (Dighe & Moudgal 1983).

Results

Generation and characterization of MAbs

MAb E12 was raised against the recombinant hCG expressed using the *Pichia pastoris* expression system. The fusion and selection of the clones secreting the antibodies were carried out as described earlier. The antibody was selected based on its ability to bind to urinary hCG by ELISA and confirmed by demonstrating binding to ^{125}I-hCG. Other MAbs used in this study have been described earlier (Dighe & Moudgal 1983, Dighe *et al.* 1990*a*).

Characterization of MAb E12

The antigen specificity of MAb E12 was determined using ELISA and confirmed by binding assays using radioiodinated hCG, hLH, hFSH, hTSH, hCGα and hCGβ. As shown in Fig. 1a and b, MAb E12 could bind hCG, both in the ELISA and in the radioiodinated hCG binding assay, but in the same experiments failed to bind hLH, clearly demonstrating that the antibody recognizes an epitope present in hCG that is completely absent in hLH. MAb E12 also failed to bind ^{125}I-hCGα, ^{125}I-hFSH and ^{125}I-hTSH, indicating that the epitope is not present in the α subunit (Fig. 1c). Similarly, it also failed to bind hCGβ in both assays, demonstrating that the MAb specifically recognizes the unique epitope present in the heterodimeric hCG (Fig. 1c). Lack of binding to the isolated β subunit also suggested that the antibody does not recognize the C-terminal portion of hCG β subunit that distinguishes it from the hLH β subunit.

The affinity of the MAb was determined by incubating ^{125}I-hCG in the presence of increasing concentrations of unlabelled hCG and the binding data obtained were converted into a Scatchard plot. As shown in the Fig. 1d, MAb E12 displayed very-high-affinity binding to hCG ($K_d = 2 \cdot 8 \times 10^{-10}$ M).

Effect of MAb on binding of ^{125}I-hCG to the receptor

MAb E12 was preincubated with ^{125}I-hCG for 1 h at room temperature followed by addition of the receptor and determination of the receptor-bound hormone. As shown in Fig. 2, MAb E12 inhibited binding of ^{125}I-hCG to the receptor, indicating that the epitope recognized by the antibody is important for hormone–receptor interaction.

Effect of antibody on response to hCG and hLH

The ability of MAb E12 to inhibit responses to hCG and hLH was next investigated using the mouse Leydig cell bioassay. As shown in the Fig. 3, MAb E12 or its ScFv could inhibit the response to hCG, but not to hLH. These data confirmed that MAb E12 recognizes the unique epitope present in hCG that is not present in hLH.

Epitope mapping

Having demonstrated the ability of MAb E12 to distinguish between hCG and hLH, three different approaches were employed to locate the epitope.

The first approach employed the strategy of carrying out RIA displacement analysis with several chemically modified derivatives of hCG, as described by Venkatesh & Murthy (1997) and Venkatesh *et al.* (1999). Different chemical modifications or enzymatic digestions of hCG were carried out under non-reducing conditions and the ability of each derivative to inhibit binding of ^{125}I-hCG to the MAb was determined. Depending on the retention of the activity as estimated by displacement analysis following a specific modification, the contribution of specific residues towards the formation of epitopes can be determined. The contribution of the specific residues is likely to be minimal if the modification results in the retention of more than 70% of the RIA displacement activity. Residues are likely to be proximal to the epitope if 20–70% of the activity is retained, while less than 20% activity means the residues are at the core of the epitope. The exact location of the epitope can be predicted taking into account the sequence and the three dimensional structure of the protein. Table 1 summarizes the modifications carried out, residues modified and the retention of the hCG-like activity by RIA with MAb E12 with ^{125}I-hCG as the probe. Binding of hCG to the antibody was completely abolished when the residues R, Y, M, H and acidic residues either D or E were chemically modified. In addition, the epitopic region seemed to be very sensitive to digestion with trypsin and chymotrypsin, confirming the contributions of the basic and aromatic residues to the epitope. Lack of effect of carboxypeptidase on the activity also confirms that the C-termini of both subunits do not contribute to the epitope. Finally, treatment with formic acid resulted in more than 80% loss in activity, confirming that the epitope is generated only when both subunits are annealed, generating the heterodimer.
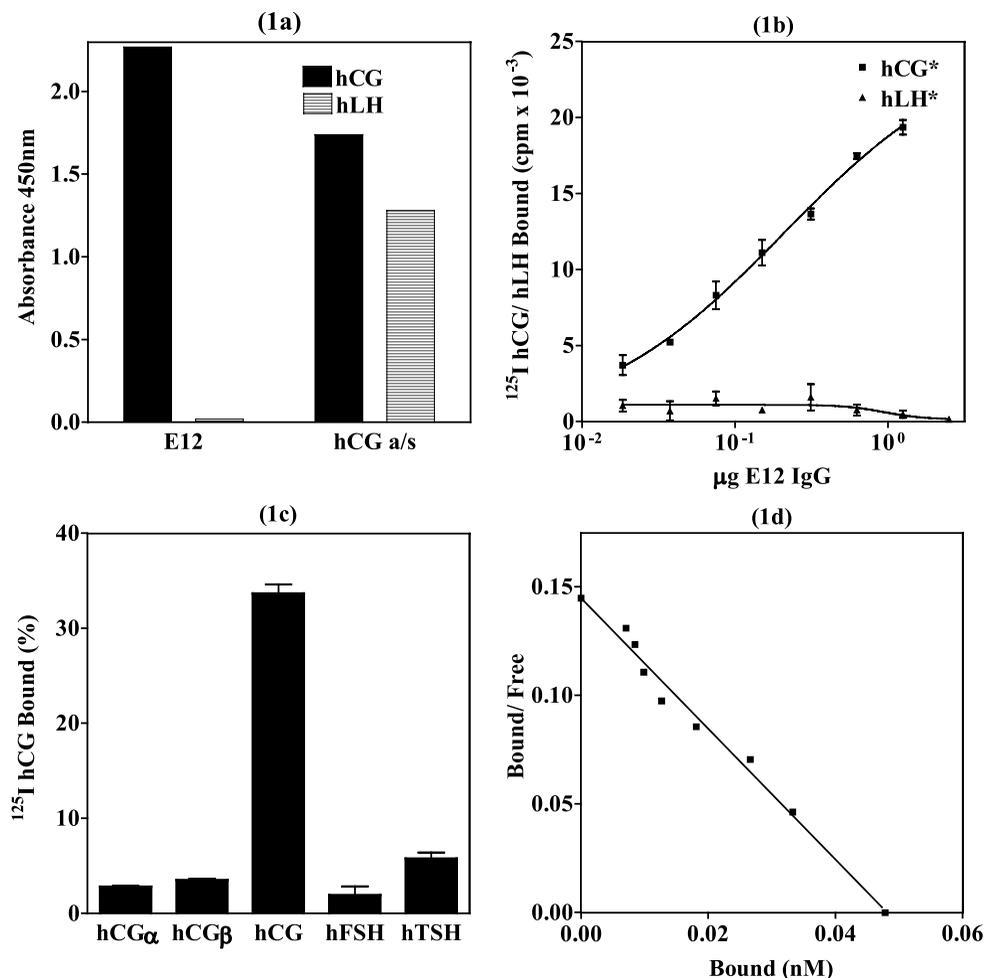
Figure 1 Characterization of E12 antibody. The antigen specificity of the antibody was determined by ELISA, as well as by RIA. The values presented are the means of duplicates and each experiment was carried out at least twice. (a) Binding of MAb E12 to hCG and hLH was carried out using ELISA with a large excess of the MAb (1 μg/well). In the same experiment, binding of both antigens to the polyclonal hCG antiserum was also determined to demonstrate the integrity of the antigens. (b) Binding of ^{125}I-hCG/hLH to increasing concentrations of MAb E12 IgG was tested by RIA. The non-specific binding was determined by incubating the radioiodinated probes in the absence of MAb E12 and was subtracted from the total binding obtained in each case. (c) Cross-reactivity of the antibody to other members of the glycoprotein hormone family, as well as the subunits of hCG was determined by RIA using ^{125}I-hCGα/hCGβ/hCG/hFSH/hTSH as probes. The data shown here represent the percentage of the total input radioactivity that was bound to the antibody in each case. (d) Binding of ^{125}I-hCG to the antibody was determined in the presence of increasing concentrations of unlabelled hCG and the binding data obtained were converted into a Scatchard plot. *n*=3–4.

The second approach was to determine binding of this MAb by ELISA to single-chain hCG and mutants of single-chain hCG expressed using the *Pichia* expression system. As shown in Fig. 4, the MAb can bind to the purified single-chain hCGαβ (Sen Gupta & Dighe 2000, R A Gadkari and R R Dighe, unpublished observations). It could also bind to hCGα[P38A]αβ and hCGα[T54A]αβ (Sunita 2003, S Setlur and R R Dighe, unpublished observations). Both these mutants can bind to the receptor and elicit biological responses.

However, the MAb failed to bind to hCGα[Q13K, E14K, P16K, Q20K]αβ (Sunita 2003, S Setlur and R R Dighe, unpublished observations), in which four lysine residues were introduced in the L1 loop of hCGα, suggesting that L1 may either contribute or influence the epitope recognized by MAb E12. Thus, binding was completely abolished when the mutations were carried in the lower half of hCG, while significant activity was retained when the mutations were carried out in the upper half of the molecule. Therefore, the binding site of
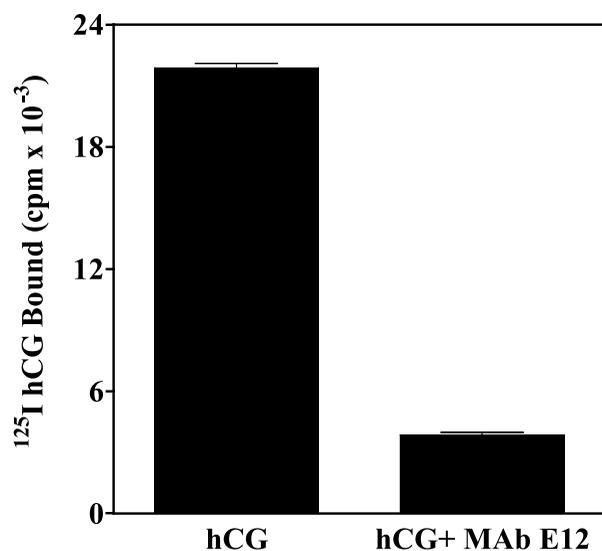
Figure 2 RRA. The effect of antibody on binding of hCG to its receptor was tested by RRA using ^{125}I-hCG as the tracer and rat testis membrane preparation as the source of LH receptors. ^{125}I-hCG (approximately 200 000 c.p.m.) was preincubated with either 1 µg MAb E12 IgG, buffer or normal mouse IgG for 1 h at room temperature. The receptor preparation was added and incubation continued for 1 h more. At the end of incubation, the receptor-bound radioactivity was determined. The non-specific binding was determined by incubating the labelled hormone with the receptor preparation in the presence of a large excess of unlabelled hCG. $n=3–4$.
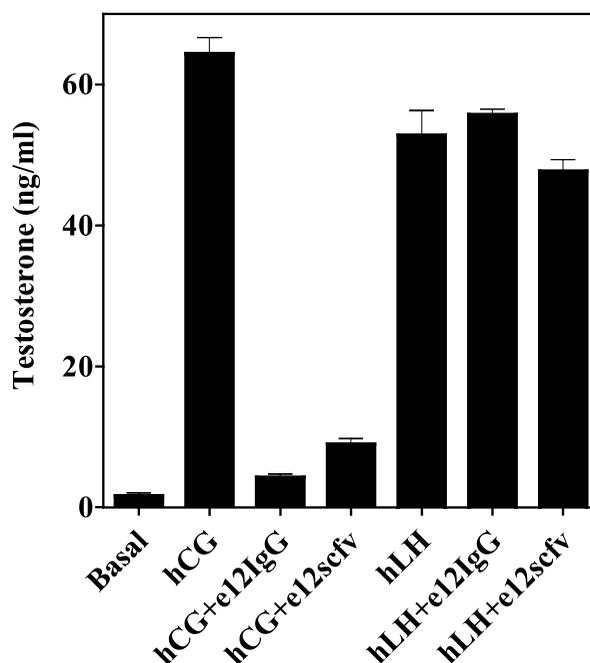

Figure 3 *In vitro* Leydig cell bioassay. Effect of antibody on response to hCG and hLH was determined by preincubating the hormones with either MAb E12 IgG or E12 ScFv expressed using the *Pichia pastoris* expression system for 1 h at room temperature followed by addition of the mouse Leydig cell preparation and continuing the incubation at 32 °C for another 4 h. Testosterone secreted into medium was determined by RIA. Values presented are means±S.D. of quadruplicates. $n=3–4$.

this antibody appears to be in the lower part of hCG (Fig. 5).

Finally, computer-aided modelling studies were carried out to arrive at the epitopic region of this antibody. The alignment between hCGβ and hLHβ sequences obtained using the 'malign' program (Martinez 1988) showed a 15% difference in the identity at the amino acid sequence level. As shown in the Fig. 6, the residues in hCGβ that are different from those in hLHβ and are solvent accessible were identified. The solvent accessibility of the residues was arrived at using the PSA program modified by A Sali (Department of Biopharmaceutical Sciences, University of California, San Francisco, CA, USA; unpublished data), which is based on the algorithm of Lee & Richards (1971). Maximum residue level differences between the two hormone subunits were found in the C-terminal region. In addition, there is a glycosylation site in hCGβ (N13) that is not present in hLHβ. Apart from these regions of hCGβ, more residue variations between hCG and hLHβ subunits were observed in the interfacial loop. These differences are encircled in Fig. 5.

Discussion

The basic premise in the development of an hCG-based female contraceptive vaccine is the generation of an antibodies that specifically neutralize hCG, resulting in termination of pregnancy, but do not react with hLH and therefore do not disrupt the cycles of the immunized women. However, this task has proved to be difficult as most of the antibodies raised against hCG or hCGβ cross-react with hLH. Numerous efforts have been made in the past to identify the epitopes in hCG that are distinct from those in hLH (Moyle *et al.* 1995, Berger *et al.* 2002), minimize cross-reaction of hCG antibodies with hLH by carrying out site-directed mutagenesis (Jackson *et al.* 1996, Porakishvili *et al.* 2002) and modulate the immunogenicity of the antigen by annealing hCGβ with oLHα (Talwar *et al.* 1994). However, it has not been possible to obtain an antibody that can neutralize hCG bioactivity while having no cross-reactivity with hLH. Therefore, an antibody that binds hCG, but has absolutely no cross-reactivity with hLH provides a unique opportunity to identify the structural differences between the two hormones.

Table 1 Effect of various chemical modifications and enzyme treatments on binding of hCG to MAb E12. Binding of ^{125}I-hCG to MAb E12 was determined in the presence of varying concentrations of hCG derivatives prepared by chemical modifications and enzyme treatments under non-reducing conditions as described earlier (Venkatesh & Murthy 1997, Venkatesh *et al.* 1999). Displacement data obtained were analysed using Graphpad Prism 4 and the activity remaining was calculated

	Residues of group modified	Activity retained (%)
Chemical		
Trinitrobenzylsulphonate	-NH$_2$ of K	1·3
Tetranitromethane	Y	2·5
Formic Acid	Subunits fall apart	20
Cyanogen bromide	C-terminal of M	0·6
Carbodiimide	D or E	3
Acetic anhydride	-NH$_2$ of K	100
Phenyl glyoxal	Guanidino of R	1·9
Diethyl pyrocarbonate	H	0·8
Enzymes		
Trypsin	K or R	0·9
Chymotrypsin	F or Y	15
Carboxypeptidase	C-terminus	70

MAb E12 was generated by immunizing a mouse with *Pichia*-expressed hCG (Gadkari *et al.* 2003), but was selected on the basis of binding to urinary hCG.
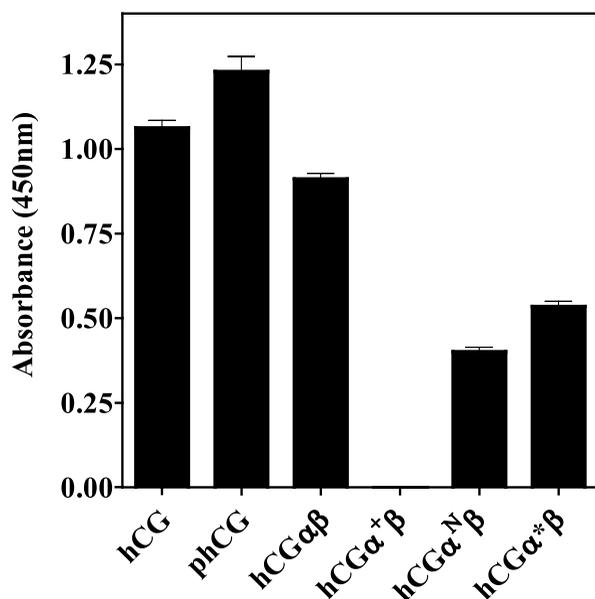

Figure 4 ELISA with mutants of hCG. Equal quantities of urinary and recombinant hCG (phCG), single-chain derivatives of hCG (hCGαβ) and its mutants were coated onto the ELISA plate and binding of MAb E12 to each one of them was determined by ELISA. The single-chain mutant hCGα^{P38A}β has been designated as hCGα*β, hCGα^{T54A}β as hCGα^{N}β and hCGα$^{Q13K,\ E14K,\ P16K,\ Q20K}$β as hCGα$^+$β. *n*=3–4.

Figure 5 Comparison of hCG and hLH. Shown in dark grey is the α subunit, while the β subunit is shown in light grey. The horizontal line marks the boundary of the upper and the lower parts of hCG. The regions in hCGβ that are different from those in hLHβ have been encircled. The diagram was constructed using the 'Setor' program (Evans 1993).

Interestingly, the antibody also does not bind either subunit of hCG and therefore recognizes a unique epitope that is generated when the two subunits combine to give rise to a heterodimeric hormone capable of binding to the receptor and eliciting a biological response. The antibody is quite intriguing as it does not recognize hLH at all, signifying that this epitope is present in hCG, but not in hLH. Further, the ability of this antibody to inhibit binding of the hormone and a response suggests that the epitope is important for the bioactivity of the hormone and therefore an ideal epitope for eliciting an immunological response that neutralizes hCG, but not hLH. The antibody failed to decrease binding of the hormone to the receptor if the latter was preincubated with the hormone, suggesting that the epitope is not accessible to the antibody when
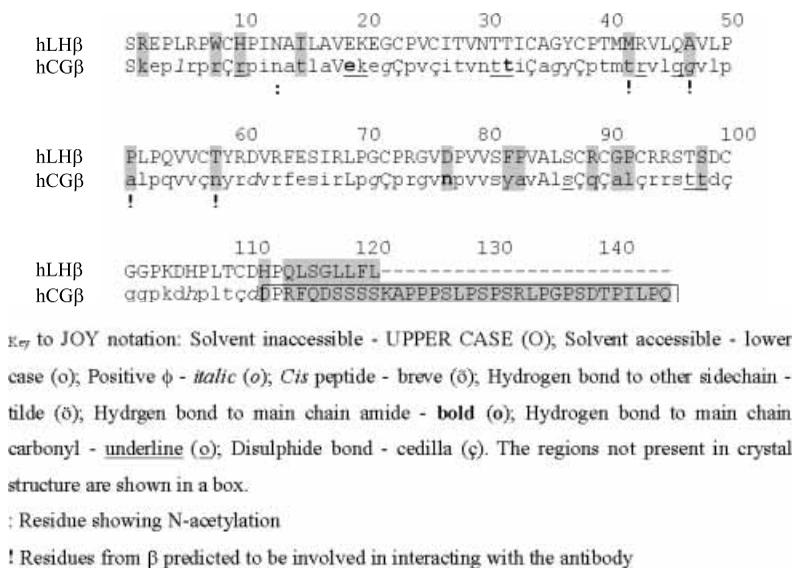
Key to JOY notation: Solvent inaccessible - UPPER CASE (O); Solvent accessible - lower case (o); Positive φ - *italic* (o); *Cis* peptide - breve (ŏ); Hydrogen bond to other sidechain - tilde (õ); Hydrogen bond to main chain amide - **bold** (o); Hydrogen bond to main chain carbonyl - underline (o); Disulphide bond - cedilla (ç). The regions not present in crystal structure are shown in a box.

: Residue showing N-acetylation

! Residues from β predicted to be involved in interacting with the antibody

Figure 6 Sequence alignment. The sequence alignment between hCGβ and hLHβ was obtained using the 'malign' program. The residues that show variations between the two hormone subunits are highlighted in grey. The residues that are at the interface are marked with '!' below. The structural environment of all the residues is represented using 'JOY' (Mizuguchi *et al.* 1998).

the hormone is complexed with the receptor (data not shown). These results were similar to those obtained with the polyclonal antisera (Dighe & Moudgal 1983).

It is evident that MAb E12 recognizes a discontinuous epitope. Exact location of such epitopes in complex molecules such as hCG is usually a very difficult task (Charrel-Dennis *et al.* 2004). We have used three different approaches to identify the MAb E12 specific epitope. The most striking difference between hCG and hLH is the C-terminal 35 amino acids present in hCGβ that are absent in hLHβ. However, the antibody is not likely to recognize this region of hCGβ as it does not bind the isolated β subunit of hCG. The sequence alignment of hCGβ and hLHβ reveals several differences between the two subunits at positions 8, 10, 15, 42, 47, 51, 58, 77, 82, 83, 89, 91, 92, 98, 112 and 114–145, the last being the CTP present in hCGβ. Based on the RIA analysis of chemically modified derivatives of hCG, it can be concluded that the residues that are likely to contribute to the epitope are methionine, arginine, aspartic acid, tyrosine, phenylalanine and histidine. In view of these data and also the differences between hCGβ and hLH, the residues that are most likely to contribute are βM41, βR43, βG47, βA51, βN58 and βD61, and αE56, αY65, αF74, αE77 and αH79. These residues are present in the L2 loop of the β subunit and the L3 loop of the α subunit respectively (Lapthorn *et al.* 1994, Wu *et al.* 1994). Some contribution from the L1 loop of the α subunit to the epitope is also possible as the antibody failed to bind single-chain

hCGα$^{Q13K, E14K, P16K, Q20K}$αβ (Sunita 2003, S Setlur and R R Dighe, unpublished observation).

Based on these data, we conclude that the epitope recognized by MAb E12 is present in the L2 loop of the β subunit and L3 loop of the α subunit (Fig. 7). In the past, one such antibody recognizing hCGβ L2 loop that binds hCG heterodimer with higher affinity as compared with the free hCGβ subunit (epitope designated as c3) has been reported by Berger *et al.* (2002). However, the exact location of the epitopic region has not been reported. Studies carried out by Schwarz and co-workers revealed that the antibodies belonging to the groups c1 and c2 are closely related to c3 group of antibodies recognizing the epitopes generated only on heterodimerization of hCG subunits and thus are similar to MAb E12 reported in the present study. These MAbs show inhibitory effects on hCG–receptor interaction similar to those shown by MAb E12 (Schwarz *et al.* 1988). We have located the epitopes of two other β subunit-specific MAbs capable of recognizing both hCG and hLH in the determinant loop of the β subunit. An α subunit-specific MAb was shown to bind hCG and hLH in the L3 loop of the α subunit (R A Gadkari and R R Dighe, unpublished observations). In contrast, MAb E12, which has unique specificity, binds hCG in the L2 of the β subunit and the L3 of the α subunit. Additional evidence in support of this argument is that the surface potential around the proposed epitopic region in hCGβ is significantly different from that in hLHβ (Fig. 8). The charge on
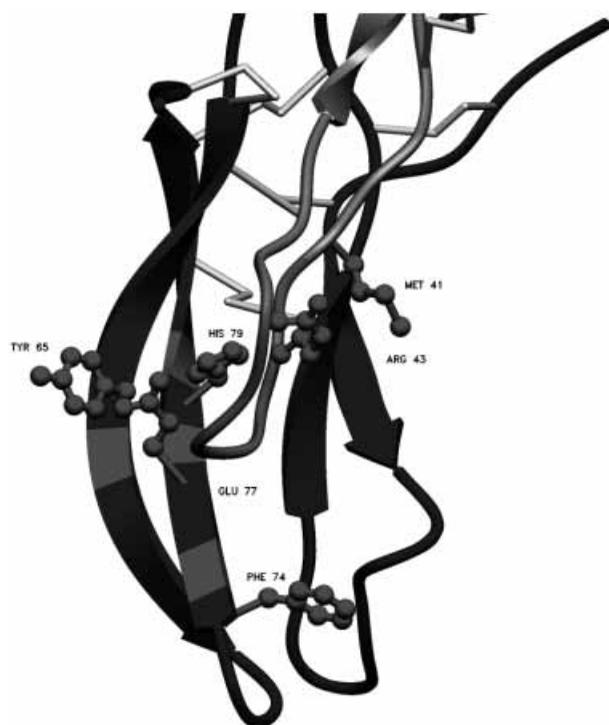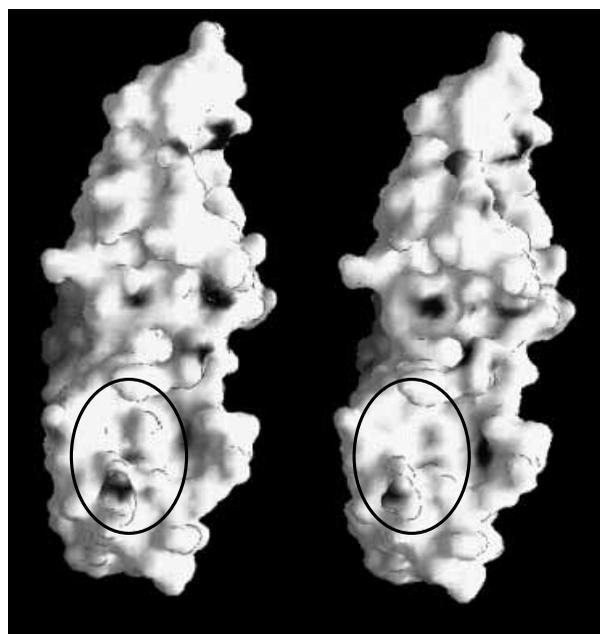
Figure 7 Epitopic region. The figure depicts the epitopic region of MAb E12. The α subunit has been shown in dark grey, while the β subunit is in light grey. The residues of both subunits identified as important for antibody binding have been shown as a 'ball and stick' model. The diagram was constructed using the 'Setor' program (Evans 1993).

hLH   hCG

Figure 8 Surface models of hCG and hLH. The surface models of hCG and hLH were generated using the 'Grasp' program (Nicholls *et al.* 1991). The proposed epitopic region of MAb E12 has been encircled in both hCG and hLH models to highlight the differences in the potentials of the two hormones in that region.

the hCG molecule in this region is more negative than that in hLH.

The difference between hCG and hLH can be exploited further for eliciting antibodies capable of specifically bioneutralizing hCG. We believe that the epitope identified in this study is the one that should be targeted for hCG-based immunocontraception. However, it will be rather difficult to generate such an antibody in humans with conventional immunization protocols used for immunocontraceptive vaccines. Interestingly, this antibody was produced using *Pichia*-expressed hCG as the immunogen. This recombinant hormone has several additional amino acids in both subunits (Sen Gupta & Dighe 1999) and has a different carbohydrate structure (Blanchard *et al.* 2003, V Blanchard, unpublished observations). However, the conformation of the hormone, as judged by immunological criteria, is similar but not identical to that of the natural hormone. Whether immunization with *Pichia*-expressed recombinant hCG would elicit antibodies that can distinguish between hCG and hLH remains to be explored.

MAb E12 can also be used for passive immunization in cases of emergency contraception. The ScFv that has

been produced in this study is functional in terms of binding the hormone and blocking its response. We have expressed this antibody using *Pichia* expression vector, established fermentation protocols with the clone producing the antibody, purified it, and are in the process of characterizing it further.

Acknowledgements

References

Berger P, Sturgeon C, Bidart JM, Paus E, Gerth R, Niang M, Bristow A, Birken S & Stenman UH 2002 The ISOBM TD-7 Workshop on hCG and related molecules. Towards user-oriented standardization of pregnancy and tumor diagnosis: assignment of epitopes to the three-dimensional structure of diagnostically and commercially relevant monoclonal antibodies directed against human chorionic gonadotropin and derivatives. *Tumour Biology* **23** 1–38.

Blanchard V, Gadkari RA, Gerrit J. Gerwig GJ, Bas R. Leeflang, Dighe RR & Kamerling JP 2003 Characterization of the N-linked oligosaccharides from human chorionic gonadotropin expressed in the methylotrophic yeast, *Pichia pastoris*. *12th European Carbohydrate Symposium*, 6–11 July, Grenoble, France.

Charrel-Dennis M, Jackson AM, Lund T, Lapthorn AJ, Berger P, Roitt IM & Delves PJ 2004 The major hormone-specific discontinuous epitopes on human chorionic gonadotrophin. *Journal of Molecular Endocrinology* **32** 571–581.

Delves PJ 2004 How far from a hormone-based contraceptive vaccine? *Journal of Reproductive Immunology* **62** 69–78.

Dighe RR & Moudgal NR 1983 Use of alpha- and beta-subunit specific antibodies in studying interaction of hCG with Leydig cell receptors. *Archives of Biochemistry and Biophysics* **225** 490–499.

Dighe RR, Murthy GS & Moudgal NR 1990*a* Two simple and rapid methods to detect monoclonal antibodies with identical epitope specificities in a large population of monoclonal antibodies. *Journal of Immunological Methods* **131** 229–236.

Dighe RR, Murthy GS, Kurkalli BS & Moudgal NR 1990*b* Conformation of the alpha-subunit of glycoprotein hormones: a study using polyclonal and monoclonal antibodies. *Molecular and Cell Endocrinology* **72** 63–70.

Dirnhofer S & Berger P 1995 Vaccination for birth control. *International Archives of Allergy and Immunology* **108** 350–354.

Dirnhofer S, Wick G & Berger P 1994 The suitability of human chorionic gonadotropin (hCG)-based birth control vaccines. *Immunology Today* **15** 455–501.

Evans S 1993 SETOR: hardware lighted three-dimensional solid model representations of macromolecules. *Journal of Molecular Graphics* **11** 134–138.

Fox HKF 1970 Immunofluorescent localisation of chorionic gonadotrophin in the placenta and in tissue cultures of human trophoblast. *Journal of Pathology* **101** 277–282.

Gadkari R, Deshpande R & Dighe RR 2003 Hyperexpression and purification of biologically active human luteinizing hormone and human chorionic gonadotropin using the methylotropic yeast, *Pichia pastoris*. *Protein Expression and Purification* **32** 175–184.

Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E & Crea R 1988 Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analog produced in *Escherichia coli*. *PNAS* **85** 5879–5883.

Jackson AM, Klonisch T, Lapthorn AJ, Berger P, Isaacs NW, Delves PJ, Lund T & Roitt IM 1996 Identification and selective destruction of shared epitopes in human chorionic gonadotropin beta subunit. *Journal of Reproductive Immunology* **31** 21–36.

Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ & Isaacs NW 1994 Crystal structure of human chorionic gonadotropin. *Nature* **369** 455–461.

Lee B & Richards FM 1971 The interpretation of protein structures: estimation of static accessibility. *Journal of Molecular Biology* **55** 379–400.

Louvet JP, Ross GT, Birken S & Canfield RE 1974 Absence of neutralizing effect of antisera to the unique structural region of human chorionic gonadotropin. *Journal of Clinical Endocrinology and Metabolism* **39** 1155–1158.

Martinez HM 1988 A flexible multiple sequence alignment program. *Nucleic Acids Research* **16** 1683–1691.

Mitchison N 1990 Gonadotropic vaccines. *Current Opinion in Immunology* **2** 725–727.

Mizuguchi K, Deane CM, Blundell TL, Johnson MS, Overington JP 1998 JOY: protein sequence-structure representation and analysis. *Bioinformatics* **14** 617–623.

Moyle WR, Campbell RK, Rao SN, Ayad NG, Bernard MP, Han Y & Wang Y 1995 Model of human chorionic gonadotropin and lutropin receptor interaction that explains signal transduction of the glycoprotein hormones. *Journal of Biological Chemistry* **270** 20020–20031.

Nicholls A, Sharpe KA & Honig B 1991 Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **11** 281–296.

Porakishvili N, Chiesa MD, Chikadze N, Martensen P, Justesen J, Lund T, Delves PJ & Roitt IM 2002 Elimination of luteinizing hormone cross-reactive epitopes from human chorionic gonadotropin. *Vaccine* **20** 2053–2059.

Schwarz S, Berger P, Nelboeck E, Khashabi D, Panmoung W, Klieber R & Wick G 1988 Probing the receptor-interaction of glycoprotein hormones with monoclonal antibodies. *Journal of Receptor Research* **8** 437–453.

Sen Gupta C & Dighe RR 1999 Hyperexpression of biologically active human chorionic gonadotropin using the methylotropic yeast, *Pichia pastoris*. *Journal of Molecular Endocrinology* **22** 273–283.

Sen Gupta C & Dighe RR 2000 Biological activity of single chain chorionic gonadotropin, hCGalphabeta, is decreased upon deletion of five carboxyl terminal amino acids of the alpha subunit without affecting its receptor binding. *Journal of Molecular Endocrinology* **24** 157–164.

Stevens VC 1996 Progress in the development of human chorionic gonadotropin antifertility vaccines. *American Journal of Reproductive Immunology* **35** 148–155.

Strauss JF III, Gafvels M & King BF 1995 *Placental Hormones*, edn 3, pp. Eds LJD *et al.* Philadelphia, PA: WB Saunders.

Sunita BR 2003 *Single Chain Glycoprotein Hormones: Structure–Function Studies*. PhD thesis. Bangalore India. Indian Institute of Science.

Talwar GP, Singh O, Pal R, Chatterjee N, Sahai P, Dhall K, Kaur J, Das SK, Suri S, Buckshee K *et al.* 1994 A vaccine that prevents pregnancy in women. *PNAS* **91** 8532–8536.

Venkatesh N & Murthy GS 1997 Immunochemical approach to the mapping of an assembled epitope of human chorionic gonadotropin: proximity of CTP-alpha to the receptor binding region of the beta-subunit. *Journal of Immunological Methods* **202** 173–182.

Venkatesh N, Krishnaswamy S, Meuris S & Murthy GS 1999 Epitope analysis and molecular modeling reveal the topography of the C-terminal peptide of the beta-subunit of human chorionic gonadotropin. *European Journal of Biochemistry* **265** 1061–1066.

Wu H, Lustbader JW, Liu Y, Canfield RE & Hendrickson WA 1994 Structure of human chorionic gonadotropin at 2·6 Å resolution from MAD analysis of the selenomethionyl protein. *Structure* **2** 545–558.