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The Human Chorionic Gonadotropin-β Arginine^{68} to Glutamic Acid Substitution Fixes the Conformation of the C-Terminal Peptide

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Wild-type human chorionic gonadotropin (hCG) has been used as a contraceptive vaccine. However, extensive sequence homology with LH elicits production of cross-reactive antibodies. Substitution of arginine^{68} of the β-subunit (hCGβ) with glutamic acid (R68E) profoundly reduces the cross-reactivity while refocusing the immune response to the hCGβ-specific C-terminal peptide (CTP). To investigate the molecular basis for this change in epitope usage, we immunized mice with a plasmid encoding a truncated hCGβ-R68E chain lacking the CTP. The animals produced LH-cross-reactive antibodies, suggesting that the refocused immunogenicity of R68E is a consequence of epitope masking by a novel disposition of the CTP in the mutant rather than a structural change in the cross-reactive epitope region. This explanation was strongly supported by surface plasmon resonance analysis using a panel of anti-hCGβ-specific and anti-hCGβ/LH cross-reactive monoclonal antibodies (mAbs). Whereas the binding of the LH cross-reactive mAbs to hCGβ-R68E was eliminated, mAbs reacting with hCGβ-specific epitopes bound to hCGβ and hCGβ-R68E with identical affinities. In a separate series of experiments, we observed that LH cross-reactive epitopes were silent after immunization with a plasmid encoding a membrane form of hCGβ-R68E, as previously observed with the soluble mutant protein itself. In contrast, the plasmid encoding the soluble secreted form of hCGβ-R68E evoked LH cross-reactive antibodies, albeit of relatively low titer, suggesting that the handling and processing of the proteins produced by the two constructs differed. (*Molecular Endocrinology* 19: 1803–1811, 2005)

HUMAN CHORIONIC gonadotropin (hCG) is a member of the glycoprotein hormone family, which also includes LH, FSH, and TSH. The glycoprotein hormones are heterodimeric molecules consisting of a common α-chain noncovalently associated with a hormone-specific β-chain. In a healthy woman, biologically active hCG is only present in significant concentrations during pregnancy where it stimulates the corpus luteum to produce progesterone and estrogen required for sustaining the implanted embryo. Incubation of marmoset embryos with hCG-specific antibodies prevents implantation (1), and hCG is considered an antifertility vaccine candidate (2–5). Phase II trials of a heterospecies vaccine consisting of an ovine α-chain and hCG β-chain coupled to tetanus toxoid or diphtheria toxoid resulted in only one pregnancy among 1224 cycles in those immunized women producing circulating anti-hCG antibodies above 50 ng per ml (2). However, due to the 85% sequence homology between the first 110 amino acid residues of hCGβ and LHβ, most of the anti-hCG antibodies produced cross-reacted with LH (3). Although no adverse effects of the LH-cross-reactive antibodies were observed in the cohort studied (3), the long-term presence of such antibodies may cause undesired side effects.

With the aim of producing a potentially safer hCG-based vaccine, we have constructed a series of hCGβ mutants in which amino acid residues at presumed LH cross-reactive epitope regions have been substituted (6). One of these mutants, hCGβ-R68E, with arginine^{68}

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* M.C.-D. and N.T. contributed equally to this work.

Abbreviations: CBB, Carbonate-bicarbonate buffer; CTP, C-terminal peptide; HBS, HEPES-buffered saline; hCG, human chorionic gonadotropin; mAb, monoclonal antibody; PBS-T, PBS containing 0.05% Tween 20; RU, resonance unit.

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substituted with glutamic acid, failed to bind all tested LH-cross-reactive monoclonal antibodies (mAbs) without apparently affecting the natural folding of the molecule, as judged by the ability of the conformation-dependent hCG-specific mAbs to recognize the protein (6). Baculovirus-produced hCGβ-R68E elicited antibodies with minimal LH cross-reactivity in both mice and rabbits (7, 8). The antibodies produced were predominantly directed against sequences present on the hCGβ-specific C-terminal peptide region (hCGβCTP; amino acids 113–145), which is missing in LHβ and has been generated during evolution by a read-through event. This peptide region adopts a free-floating entropy-rich conformation in hCG (9) and consequently is poorly immunogenic (7, 8). We hypothesized that glutamic acid[68] in the mutant forms a salt bridge with one or more of the basic amino acid residues present in the hCGβCTP, thus providing the hCGβCTP with a defined conformation that creates a new and more immunogenic loop.

We have used DNA immunization and analysis by surface plasmon resonance to further probe the structure of hCGβ-R68E. We show here that truncation of the CTP element of hCGβ-R68E (hCGβ-R68E-ΔCTP) restores the immunodominance of the LH-cross-reactive epitope region so that mice immunized with a plasmid expressing hCGβ-R68E-ΔCTP produce antibodies that recognize hCG, LH, and hCGβ-R68E equally well. Biosensor analysis is often used to determine the thermodynamics of antibody-antigen interactions. Even subtle changes in the structure of the antigen can have profound consequences for the kinetics of formation or dissociation of the protein-protein complexes. Optical biosensor analysis of the conformational integrity of hCGβ-R68E confirmed that whereas LH-cross-reactive epitopes were inaccessible in the mutant, hCGβ-specific epitopes adjacent to the cross-reactive epitope region were unaffected by the substitution. This is consistent with our proposed model postulating formation of a salt bridge between glutamic acid[68] and a basic residue on the CTP, which masks the LH-cross-reacting epitopes. The identity of the charge on CTP remains to be determined, but both K^{122} and R^{133} contribute to the formation of the loop structure.

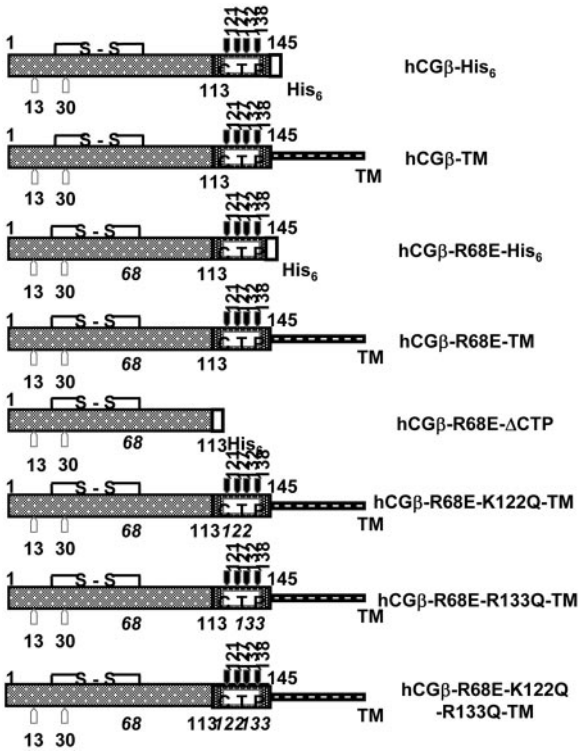

Fig. 1. Diagram of the hCGβ-Related Immunogens Used

The N-linked and the O-linked glycosylation sites are indicated with an *open* and a *solid arrow*, respectively. The numbering of amino acid residues relates to hCGβ, and the positions of the mutated residues are indicated in *italic*.

RESULTS

Immunization

BALB/c mice immunized with expression plasmids encoding either membrane-attached (pCDNA3-hCGβ-TM) or soluble (pCDNA3-hCGβ-His_6) forms of hCGβ (Fig. 1) produced antibodies that bind equally well to holo-hCG, recombinant hCGβ produced in baculovirus (Bac-hCGβ-WT), and the mutant hCGβ-R68E produced in baculovirus (Bac-hCGβ-R68E) and cross-reacted with LH (Fig. 2). This contrasts with the results obtained after immunization with pCDNA3-hCGβ-R68E. As expected, mice immunized with DNA encoding a membrane-attached form of hCGβ-R68E (pCDNA3-hCGβ-R68E-TM) produced antibodies that bind well to Bac-hCGβ-R68E. The sera from these mice reacted poorly with holo-hCG and the free Bac-hCGβ subunit and showed no cross-reactivity with LH (Fig. 2), consistent with the results obtained after intranasal immunization of mice with purified Bac-hCGβ-R68E mixed with *Escherichia coli* heat-labile protein (7). This contrasts with the result obtained in mice immunized with pCDNA3-hCGβ-R68E-His_6 encoding a secreted form of hCGβ-R68E, which resulted in antibodies that bind to Bac-hCGβ but less so to holo-hCG and LH although they reacted more strongly with Bac-hCGβ-R68E.

This is consistent with our proposed structure of hCGβ-R68E, which envisages the normally entropy-rich hCGβCTP becoming conformationally fixed by formation of a salt bridge between glutamic acid[68] and one or more of the basic residues present in the hCGβCTP (8, 10). This model predicts that mice immunized with a truncated hCGβ-R68E molecule without CTP (hCGβ-R68E-ΔCTP) would produce antibodies that bind to hCG, and notably LH, equally well as to hCGβ-R68E itself. As shown in Fig. 2, mice immunized with pCDNA3-hCGβ-R68E-ΔCTP did elicit antibodies that bind equally well to holo-hCG, LH, and Bac-hCGβ. Although the antigen-specific IgGs react stron-

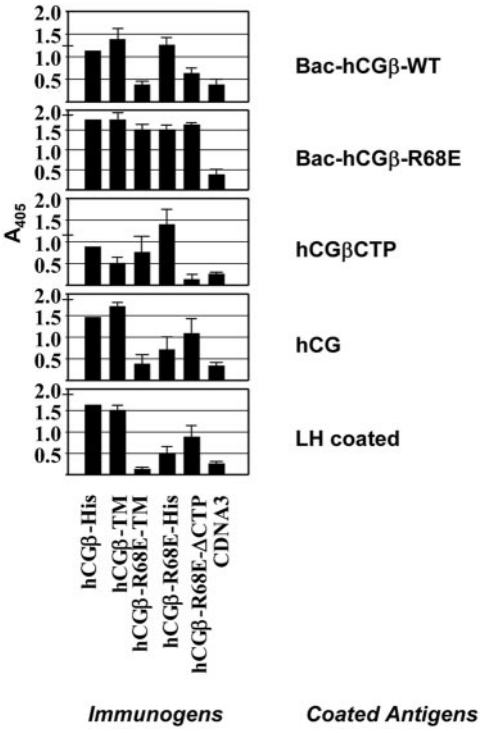

Fig. 2. The Level of hCG-Specific Antibodies after Immunization with Plasmid DNA

Female BALB/c mice (n = 5) were immunized with 50 μg plasmid DNA expressing membrane-attached (TM) or secreted (His) hCGβ or hCGβ-R68E or truncated hCGβ-R68E (hCGβ-R68E-ΔCTP) devoid of the CTP. Sera (dilution 1:50) were analyzed in ELISA using plates coated with proteins as indicated, and the results are expressed as optical density ± SD.

ger with Bac-hCGβ-R68E, there was no statistical difference [$P = 0.11$, paired Student's *t* test (LH coating *vs.* Bac-hCGβ coating); $P = 0.09$, paired Student's *t* test (LH coating *vs.* recombinant hCG coating)] between the results obtained with Bac-hCGβ, holo-hCG, or LH. This supports the hypothesis that the CTP attains a conformation in hCGβ-R68E, which blocks the accessibility of cognate B cells to the cross-reactive epitope region on the body of the hCGβ molecule.

Thermodynamic Analysis

To probe further into the conformation of hCGβ-R68E, we used surface plasmon resonance to determine the association and dissociation rates and affinity constants of selected hCG-reactive mAbs, because even modest structural changes in the overall conformation of the core domain of hCGβ as a result of the glutamic acid[68] substitution would affect the thermodynamic properties of the antibodies' binding. For this analysis, a panel of mAbs directed to LH-cross-reactive and hCGβ-specific epitopes (11, 12) were selected (Table 1). INN-22 and INN-58 have specificity for the immunodominant LH-cross-reactive epitopes, β2 and β5 (12), respectively. As representative for the hCGβ-specific mAbs, INN-2, INN-64, and INN-68, which bind to β1, β6, and β7 (12), respectively, were selected. Epitope β1 is accessible on holo-hCG and includes among its contact residues arginine[10] and arginine[60] close to the cystine knot (13), whereas β6 and β7 are both located in the interface between the α- and β-subunit and are inaccessible on holo-hCG (12). Epitope β6 is spatially close to lysine[20], glutamic acid[21], glycine[22], and glycine[75] (6), whereas epitope β7 is affected by mutations of the residues in position 61 and 89 (13).

Table 1. Specificities of the mAbs Used in the Present Study

mAb	Epitope	Epitope Properties
INN-2	β1	hCG specific
INN-22	β2	hCG/LH cross-reactive
INN-58	β5	hCG/LH cross-reactive
INN-64	β6	hCG specific (αβ-interface)
INN-68	β7	hCG specific (αβ-interface)

To reduce cross-linking by the bivalent IgGs, Fab fragments of each mAb were generated by papain digestion. The Fab fragments obtained from the individual Igs contained limited but varying amounts of undigested Igs in the samples. Although the calculated equilibrium constant therefore cannot be interpreted as an absolute value, the data obtained for wild-type mutant hCGβ can nevertheless be compared, because the same Fab/antibody ratio was used for both proteins.

Figure 3 shows the sensorgrams for the binding of INN-2, INN-22, INN-58, INN-64, and INN-68 to Bac-hCGβ-WT and Bac-hCGβ-R68E, and the resulting association rate constant (k_a), dissociation rate constant (k_d), and apparent dissociation equilibrium constant K_D are summarized in Table 2. The five Fabs recognized Bac-hCGβ-WT with a dissociation equilibrium constant varying between 8.2×10^{-8} M for INN-2 and 0.7×10^{-8} M for INN-68 Fab. This compares with the affinities of the mAbs determined by saturation or competitive RIA (14, 15). This demonstrates that the recognition of the five spatially distinct antigenic sites on Bac-hCGβ-WT by these antibodies has not been affected dramatically by production of the hCGβ subunit in insect cells, indicating that differences in post-translational modifications, such as glycosylation, in insect cells do not substantially modify the overall three-dimensional structure of the protein.

The glutamic acid[68] substitution in Bac-hCGβ-R68E abrogates the binding of the LH-cross-reactive Fabs INN-22 and INN-58, whereas binding of the Fabs to the hCG-specific epitopes, β1, β6, and β7, was unaffected by the mutation (Fig. 3 and Table 2) in agreement with the results obtained by flow cytometric analysis of transiently transfected COS-7 cells (6). At the same concentration at which the cross-reactive Fabs (INN-22 and INN-58) reacted with Bac-hCGβ-WT, minimal binding to Bac-hCGβ-R68E was observed.

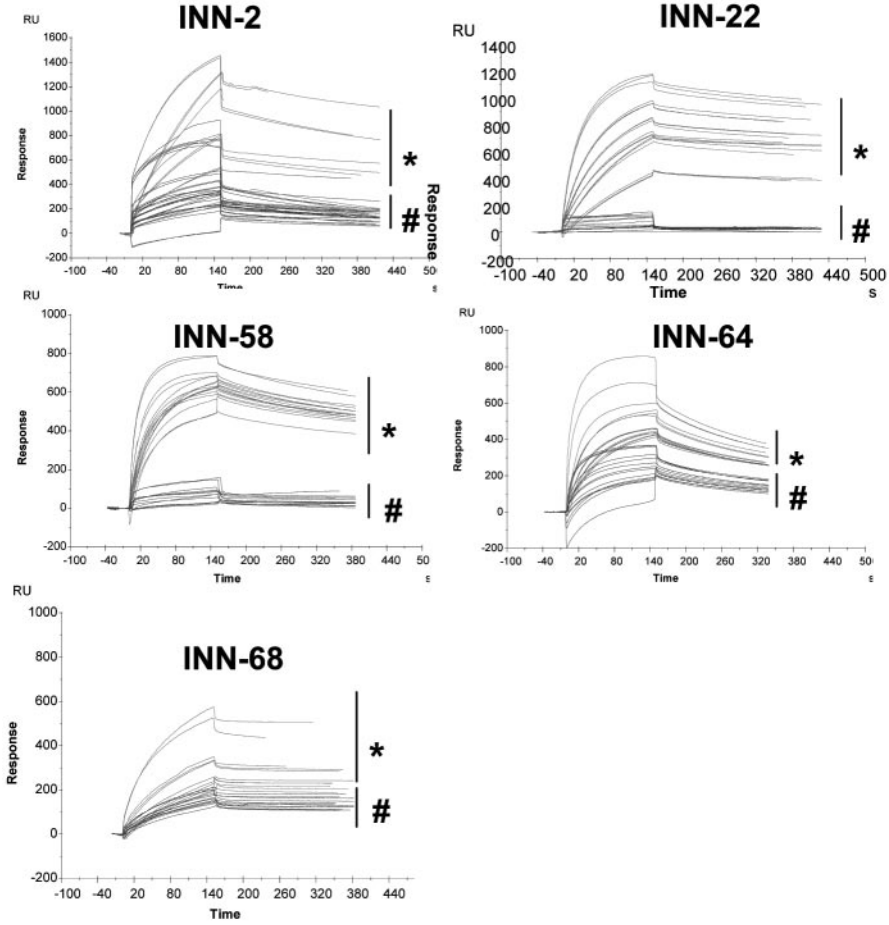

Fig. 3. Sensorgram Showing the Binding of Fabs to Bac-hCGβ or Bac-hCGβ-R68E

Recombinant Bac-hCGβ or Bac-hCGβ-R68E was bound to Ni^{2+}-charged nitrilotriacetic acid sensor chips at a concentration equivalent to 500 RU. A solution (50 μl) containing a predetermined dilution of the appropriate Fab (INN-2; INN-22; INN-58; INN-64; and INN-68) was passed onto the chip at a rate of 20 μl/min. The sensorgrams were corrected for the constant release of His-tagged recombinant protein, normalized, and overlaid. *, Sensorgrams obtained with Bac-hCGβ; #, sensorgrams obtained with Bac-hCGβ-R68E.

Indeed, the association rates of the two cross-reactive Fabs were very similar to that obtained for the isotype-matched control antibody (data not shown). In contrast, the β6 (INN-64) and β7 (INN-68) epitopes located at the interface between the α- and β-subunit remained unchanged by the mutation. The association and dissociation rate constants for the binding of each of these antibodies to Bac-hCGβ-WT and Bac-hCGβ-R68E were not statistically different (Table 2). Both β6 and β7 are spatially relatively adjacent to the cross-reactive epitope region recognized by INN-22 and INN-58. Thus the fact that the binding affinities of INN-64 and INN-68 are unaffected by the glutamic acid[68] substitution implies that the overall structure of hCGβ is maintained.

The β1-specific INN-2 antibody was previously thought to recognize the wild-type and mutant protein equally well (6). The biosensor analysis showed, however, a slightly higher association rate ($k_a = 2.5 \times 10^4\ M^{-1}\ sec^{-1}$) for INN-2 binding to the mutant protein than that observed for the binding to Bac-hCGβ-WT ($k_a = 1.5 \times 10^4\ M^{-1}\ sec^{-1}$). Nonetheless, the complex formed with the mutant protein appeared to be less stable with a $k_d = 2.7 \times 10^{-3}\ sec^{-1}$ for Bac-hCGβ-R68E compared with $k_d = 1.8 \times 10^{-3}\ sec^{-1}$ for the wild-type form of hCGβ (Table 2). Overall, the affinity of binding was significantly greater for the wild-type protein ($K_D = 8.2 \times 10^{-8}$ M) compared with that of the mutant ($K_D = 11.1 \times 10^{-8}$ M; $P < 0.001$). Nevertheless, the difference in free energy changes between INN-2 binding to the mutant and the wild-type protein was minimal ($\Delta\Delta G = 0.163$ kcal/mol). The binding of INN-2 to hCG involves residues arginine[10] and arginine[60] (13). It seems unlikely, however, that an amino acid substitution localized more than 30Å away from the β1 epitope, which does not modify the conformation of more proximal epitopes, could affect the recognition pattern of this epitope. However, the formation of a salt bridge between the glutamic acid[68] and the basic residues of the hCGβCTP could affect the spatial ori-

Table 2. Kinetic Parameters for Reaction of Fab Fragments of the INN-2, INN-22, INN-58, INN-64, and INN-68 Antibodies with Bac-hCGβ-WT and Bac-hCGβ-R68E

Antibody	Antigen	Apparent K_D (10^{-8} M)	k_a (10^4 M^{-1} sec^{-1})	k_d (10^{-3} sec^{-1})	WT/R68E[a]	ΔG^b (kcal/mol)	$\Delta\Delta G^c$ (kcal/mol)
INN-2 Fab	WT	8.2 ± 1.1[d]	1.5 ± 0.2	1.8 ± 0.37	$P = 0.0003$[a]	−9.6	0.163
	R68E	11.1 ± 2.1	2.5 ± 0.6	2.7 ± 0.5		−9.4	
INN-22 Fab	WT	1.8 ± 0.4	3.6 ± 0.7	0.6 ± 0.05		−10.5	
	R68E	N.A.	N.A.	N.A.			
INN-58 Fab	WT	1.1 ± 0.2	12.1 ± 1.9	1.3 ± 0.1		−10.8	
	R68E	N.A.	N.A.	N.A.			
INN-64 Fab	WT	4.4 ± 1.1	6.6 ± 1.4	2.8 ± 0.2	$P = 3.7$	−10.0	0.002
	R68E	4.2 ± 1.6	5.4 ± 1.1	2.3 ± 0.1		−10.0	
INN-68 Fab	WT	0.7 ± 0.2	2.8 ± 0.6	0.2 ± 0.04	$P = 0.12$	−11.0	0.162
	R68E	1.1 ± 0.3	2.4 ± 0.6	0.2 ± 0.1		−10.9	

N.A., Not ascertainable because binding is comparable to isotype control Fab and therefore too low to permit calculation of binding constants; WT, wild type.

[a] Student's *t* test was used to calculate the *P* value for K_D.

[b] The variation in free energy upon binding was determined using: $\Delta G = -RT \ln(k_a/k_d)$.

[c] The difference in free energy changes between the mutant and the wild-type protein were calculated as: $\Delta\Delta G = \Delta G_{R68E^-} \Delta G_{WT}$.

[d] ± SD.

entation of the oligosaccharide antennae, resulting in an increase in the association rate of INN-2. The increased instability of the complex formed with the mutant protein could be related to some steric hindrance of the antibody by the fixed CTP of hCGβ-R68E.

CTP Mutations

Our model predicts that the presence of an acidic residue (glutamic acid) at position 68 of hCGβ changes the chemical characteristic of this region of the protein structure. Normally this region of the structure is flat with two protruding amino acid residues, R68 and R74, approximately 10Å apart, both with a net positive charge. The substitution R68E changes both the shape and charge of this area of hCGβ, which is part of the cross-reactive epitope, although it is unlikely to greatly modify the folded structure of the protein that is tethered by the disulfide C^{23}-C^{72}. A working hypothesis for the masking of the cross-reactive epitopes is that R68E forms a salt bridge with one or more of the basic amino acid residues in the CTP. There are three possible candidate residues, R^{114}, K^{122}, and R^{133} (Fig. 4). Both R^{114} and R^{133} have negatively charged residues in relative close proximity within the peptide, which could form additional charged interactions with R^{74} on the same surface. R^{133} is an unlikely candidate as it forms part of the epitope for mAb OT3A (amino acids 133–139) the binding of which has been shown to be unaffected in the R68E mutant (6). R^{114} is also in doubt if the β-subunit disulfide C^{26}-C^{110} is formed as this would tether the CTP on the opposite side of the β-subunit and R^{114} would be too far away from R68E to form a salt bridge. However if this disulfide were not formed (due to the absence of the α-subunit), then the flexibility of the "seat belt" region of the β-subunit would probably permit R^{114} to form a salt bridge. In an attempt to identify the putative donor residue for the postulated salt bridge with R68E, we selectively substituted K^{122} and R^{133} on hCGβ-R68E with glutamine individually or together (Fig. 1) and immunized groups of BALB/c mice with plasmids precipitated onto gold particles. As shown in Fig. 5, the strongly CTP-focused and reduced hLH cross-reactive antibody response obtained with pCDNA3-hCGβ-R68E-TM was not obtained after immunization with any of the three CTP mutants: pCDNA3-hCGβ-R68E-K122Q-TM, pDNA3-hCGβ-R68E-R133Q-TM, or pCDNA3-hCGβ-R68E-K122Q-R133Q-TM. The immune response with each of the CTP mutants was similar to that obtained after immunization with pCDNA3-hCGβ-TM. This confirmed that the conformation of CTP is fixed in hCGβ-R68E but that both K^{122} and R^{133} contribute to establish this conformational change.

DISCUSSION

A single arginine[68] to glutamic acid substitution in hCGβ dramatically alters the antigenicity and immunogenicity of the mutant molecule. The immunodominant LH cross-reactive epitope cluster on hCG has been defined by several mAbs and comprises several epitopes (β2, β3, β4, and β5) (12) that may share one or more contact residues as we have previously suggested (16). Representative mAbs defining each of these epitopes fail to bind to recombinant hCGβ-R68E expressed in either COS7 cells (6) or insect cells (Table 2). We have identified, however, one CTP-reactive mAb (2F4/3) that reacts more avidly with hCGβ-R68E than with hCG or hCGβ (7), suggesting that the glutamic acid[68] substitution affects the conformation of the normally entropy-rich, free-floating CTP. Indeed, immunogenicity studies with baculovirus-derived

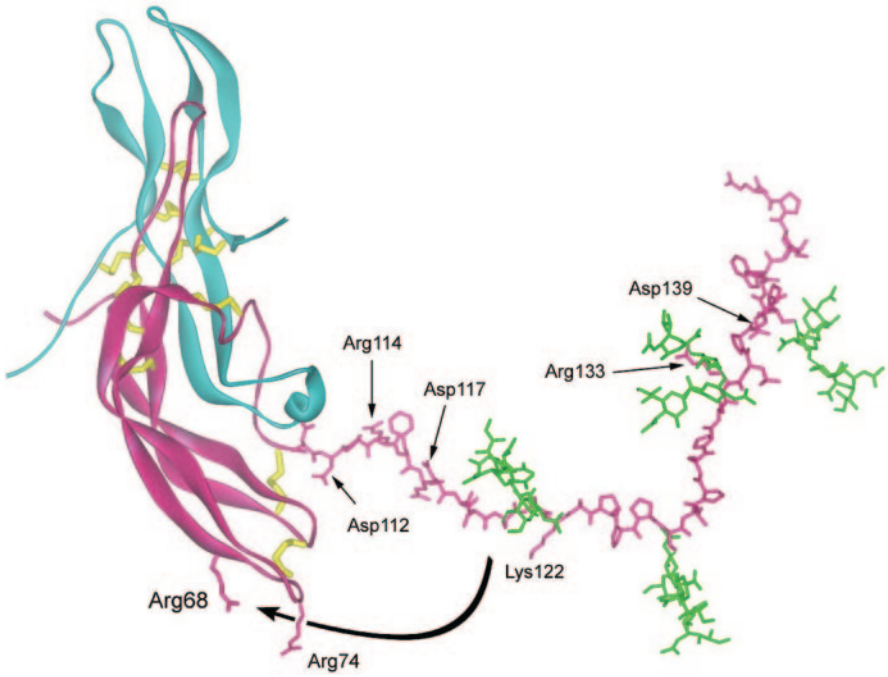

Fig. 4. A Ribbon Representation of a Molecular Model of hCG (Based on Protein Data Base Entry 1 HRP) with the α-Subunit (*Cyan*), β-Subunit (*Magenta*), and Disulfide Bridges (Shown in *Yellow*)

A chemically sensible conformation of the CTP (not seen in the x-ray structure) is modeled with O-linked carbohydrates shown in *green*. The CTP is able to wrap around the β-subunit in the R68E mutant forming an interaction that blocks the LH cross-reactive epitope. Charged amino acid residues of the CTP are labeled.

hCGβ-R68E in both mice (7) and rabbits (8) show that the immune response is refocused toward epitope(s) present on the CTP at the expense of the major LH cross-reactive epitope normally used by hCG/hCGβ as also demonstrated in the present study after DNA immunization of mice with pCDNA3-hCGβ-R68E-TM or pCDNA3-hCGβ-R68E-His_6 (Figs. 2 and 5).

To explain these findings we have proposed that the presence of an acidic residue (glutamic acid) at position 68 of hCGβ changes the chemical characteristic of this region of the protein structure. The mutation R68E could potentially permit the formation of an internal salt bridge between the glutamic acid and a positive charge associated with the CTP, thereby fixing the structure of the CTP by forming a new loop structure. Elimination of the CTP on the hCGβ-R68E immunogen (hCGβ-R68E-ΔCTP) elicited antibodies that reacted equally well with hCG, LH, and Bac-hCGβ-R68E in contrast to antibodies produced after immunization with pCDNA3-hCGβ-R68E-TM or pCDNA3-hCGβ-R68E-His_6. The presence of a new loop structure is further supported by real-time binding kinetics of mAbs to wild-type and mutant Bac-hCGβ using surface plasmon resonance. Whereas the β2- and β5-specific LH cross-reactive mAbs fail to bind Bac-hCGβ-R68E, the mAbs INN-64 and INN-68, specific for epitopes β6 and β7, which include residues located both on loop 1 and loop 3 of the β-subunit (6), bind to the wild-type and the mutant subunit with nearly identical association and dissociation rates (Table 2). This demonstrates that glutamic $acid^{68}$ does not introduce major structural changes that affect the relative spatial arrangement of loops 1 and 3, which present these two epitopes. Rather, it seems more probable that a salt bridge might be formed between glutamic $acid^{68}$ and one or more of the basic residues on CTP (K^{122} and R^{133}), thereby masking the entire cross-reactive epitope cluster and eliminating their accessibility for both isolated LH-cross-reactive mAbs and potential B cell receptors with specificity for this region of hCG. However, both K^{122} and R^{133} appear important for establishing this additional loop structure, because selectively substituting either of these residues with a noncharged glutamine abolished the CTP-loop structure of hCGβ-R68E. It is surprising that both these mutants have an effect, ruling out a simple salt bridge between E^{68} and one of the residues. Both K^{122} and R^{133} occupy rather similar positions in sequence (*i.e.* both follow a serine that is O-linked glycosylated in the CTP), so substituting either of these residues with the neutral glutamine may alter the O-linked glycans on the CTP. Equally, these residues may be important in fixing the CTP structure in hCGβ-R68E but not directly via an interaction with residue 68. Clearly, a more detailed structural analysis of hCGβ-R68E is required to determine the structure of the CTP in this mutant protein and the role of the individual amino acids in interactions with the main body of hCGβ.

Regarding antibody recognition of the other, non-CTP but hCGβ-specific, epitopes we noted less binding (at a molar basis) of the panel of mAbs to Bac-

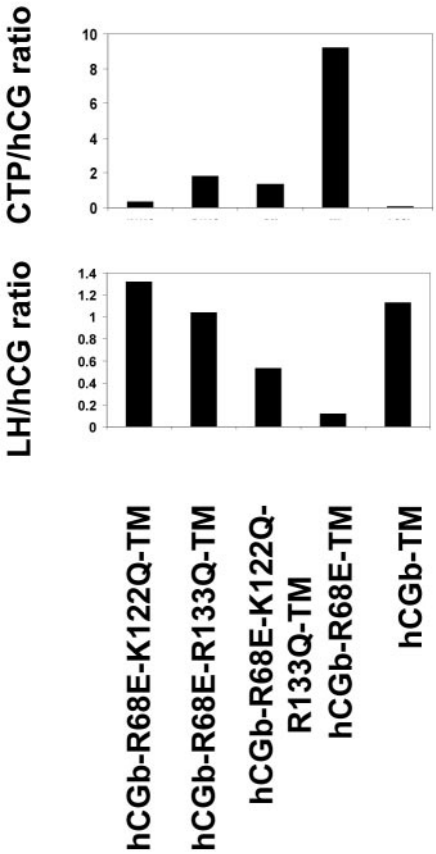

Fig. 5. The Relative Levels of Specific Antibodies after Particle-Mediated DNA Immunization with hCGβ-R68E that Contained CTP Substitutions

Female BALB/c mice (n = 5) were primed and boosted 4 wk later with 2 μg plasmid DNA coated onto 2 mg gold particles. Sera (dilution 1:50) were analyzed in ELISA using plates coated with recombinant hCG, LH, and CTP, and the ratio of mean optical density values for CTP and hCG (*upper panel*) and for LH and hCG (*lower panel*) were plotted.

hCGβ-R68E than to wild-type Bac-hCGβ. Although it is unclear why this should occur, given that hCGβ and Bac-hCGβ have a tendency to form aggregates, it is possible that the constrained conformation of the CTP in hCGβ-R68E favors structures that make the other β-specific epitopes less accessible on the mutant molecule than on the wild-type Bac-hCGβ molecule.

Depending on the antigen, mice DNA-immunized with a membrane-attached molecule can produce a greater antibody response compared with mice immunized with plasmids encoding soluble forms of the antigen, as shown, for example, with influenza virus hemagglutinin (17). This is not apparent for relatively weak antigens such as hCGβ, as shown in Fig. 2, where the membrane-attached (pCDNA3-hCGβ-R68E-TM) and soluble (pCDNA3-hCGβ-R68E-His_6) forms of the immunogen produce comparable R68E mutant-specific antibodies. However, immunization of mice with these two immunogens resulted in differences in both the magnitude and complexity of the antibodies reacting with related antigens (Fig. 2). Whereas mice immunized with pCDNA3-hCGβ-R68E-TM predominantly used epitopes specific to hCGβ-R68E, consistent with the results obtained after intranasal immunization of mice with Bac-hCGβR68E (7), immunization with pCDNA3-hCGβ-R68E-His_6 produced a higher level of antibodies reacting with hCG and hCGβ and, to a lesser extent, LH (Fig. 2). The difference in the response to immunization with the soluble mutant plasmid, as compared with the membranous form and indeed the soluble mutant protein itself, presumably reflects differences in the accessibility of the LH cross-reacting epitopes to the cognate B cell receptors as a result of some variation in the handling or processing of these immunogens by antigen-presenting cells.

MATERIALS AND METHODS

Immunization

Plasmid pCDNA3 (Invitrogen, Paisley, Scotland, UK) was used as the backbone plasmid into which the majority of the hCGβ fragments were subcloned. Fragments encoding hCGβ and hCGβ-R68E fused with the transmembrane and cytoplasmic tail of H2-D^b (6) were subcloned as *Hin*dIII-*Not*I fragments to generate pCDNA3-hCGβ-TM. pCDNA3-hCGβ-His_6 and pCDNA3-hCGβ-R68E-His_6 were constructed by subcloning of a *Hin*dIII-*Not*I hCGβ-His_6 fragment from pBac-hCGβ-WT and pBac-hCGβ-R68E-His_6 (7). pCDNA3-hCGβ-R68E-ΔCTP was made by a PCR amplification using the 5′-T7 primer (5′-TAATACGACTCACTATAGGG-3′) and 3′-primer (5′-TGCTCTAGATTAGTGGTGGTGGTGGTGGTGGT-CGACCAAGGGGTGGTCCTTGGG-3′) and subcloning the PCR product digested with *Hin*dIII and *Sal*I into the *Hin*dIII-*Sal*I-digested pLitmus-hCGβ-His_6 and subsequently subcloned into pCDNA3 as a *Hin*dIII-*Not*I fragment (Fig. 1).

pCDNA3-hCGβ-R68E-K122Q-TM and pCDNA3-hCGβ-R68E-R133Q-TM were made by PCR amplifications using 5′-M13-reverse sequence primer (5′-AGCGGATAACAATT-TCACACAGGA) and for the 3′-primers (for the K122E substitution: 5′-GATGGGCTTGGAAGGCTGGGGGGAGGGGCTT-GTGAGGAAGAGGAGTCCTGGAAG followed by a second PCR using the 3′-primer: 5′-CCAGCGTCCTCGAGTTGTGGGAG-GATCGGG), (for the R133Q substitution: 5′-TAACGCCAGCT-GTTGTGGGAGGATCGGGGTGTCTGAGGGCCCCGGGAGTT-GGGATGGACTTGGAAGGCTG followed by a second PCR with the 3′-primer 5′-CCAGCGTCCTCGAGTTGTGGGAGGATCGGG) using pLitmus-hCGβ-R68E-His_6 as template. pCDNA3-hCGβ-R68E-K122Q-R133Q-TM was made from hCGβ-R68E-K133Q with the same 3′-primers used to make hCGβ-R68E-R133Q. The final PCR products were digested with *Eco*RI and *Xho*I and subcloned into pLitmus containing the H2-K^b TM region used previously (6). The recombinant gene was finally subcloned into pCDNA3 as a *Hin*dIII-*Not*I fragment. All plasmids used to immunize mice were purified using a QIAGEN endotoxin-free purification kit (QIAGEN, Crawley, West Sussex, UK).

Female BALB/c mice (6 wk old) in groups of five were immunized one to three times. For DNA immunization the recipient animals were injected im with 6.8 μg cardiotoxin (Sigma, Poole, Dorset, UK) in 100 μl of 0.15 M PBS (pH 7.4) 6 d before being primed (wk 0) with 50 μg plasmid DNA injected into the cardiotoxin-treated muscles, and an additional boost of 50 μg plasmid DNA was given 2 wk later. After the injection of the plasmid DNA into the quadriceps, the muscles were stimulated electrically as described by

Mathiesen (18). Briefly, silver rod electrodes were placed on the skin at the site of DNA injection, and the muscles were subjected to eight trains of 1000 pulses delivered at a frequency of 1000 Hz using a Hear 6-bp stimulator (Frederick Hear, Bowdoinham, ME). Each pulse lasted 400 μsec. The electric field strength was approximately 50 V over 3–4 mm, and each train was delivered at 2-sec intervals with each train lasting 1 sec.

For particle-mediated DNA delivery the plasmids (2 μg/mg gold) were precipitated onto 2-μm diameter gold particles (DeGussa Metals Group, South Plainfield, NJ) in the presence of 0.05 M spermidine (Sigma) and 1 M calcium chloride. The gold particles were washed three times in absolute alcohol containing 0.15 M polyvinylpyrrolidone (Sigma), and then adsorbed to the inner surface of Tefzel tubing (TFX Medical, Inc., Jaffrey, NH) by centrifugal force. The Tefzel tubes were subsequently cut into 1.27-cm long cartridges and stored desiccated at 4 C. A cartridge contains 0.5–0.75 μg plasmid DNA/0.5 mg gold particles. The DNA-coated gold particles were delivered from cassettes at 500 pounds/square inch of pressure at two sites of shaved abdomen (1.0–1.5 μg total DNA) by particle-mediated DNA delivery using the Helious Gene Gun (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Mice were immunized at wk 0 and received a boost 4 wk later.

At the end of the experiment, the animals were killed by exsanguination. All animal experiments were carried out according to United Kingdom Home Office guidelines.

Immunoassays

MaxisorpC plates were coated at 4 C overnight with recombinant hCG (Sigma), hLH purified from pituitary (a kind gift from Dr. A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA), and Bac-hCGβ and Bac-hCGβ-R68E purified as described (7), at 1.0 μg/ml with 50 μl/well in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (CBB). The plates were washed three times in PBS containing 0.05% Tween 20 (PBS-T), followed by blocking with 2% dried skimmed milk powder in CBB overnight at 4 C. After washing three times with PBS-T, 50 μl serum (from the immunized or nonimmunized mice) diluted 100 times in PBS-T was added and incubated for 2 h at 37 C. The plates were washed three times with PBS-T before goat antimouse IgG alkaline phosphatase-conjugated polyclonal antibodies (Sigma) were added for 2 h at 37 C. The substrate *p*-nitrophenylphosphate in CBB containing 2 mM $MgCl_2$ was added, for 15 min, and the plates were read at A_{405} using an MR5000 ELISA plate reader (Dynatech Laboratories Ltd., Billinghurst, Sussex, UK).

Production and Purification of Baculovirus-Produced hCGβ

The construction of pBac2-hCGβ-WT and pBac2-hCGβ-R68E encoding wild-type hCGβ (Bac-hCGβ-WT) and mutant hCGβ-R68E (Bac-hCGβ-R68E) and their viruses has been described previously (7). Briefly, Bac-hCGβ-WT and Bac-hCGβ-R68E were both extended at the carboxy terminus with a His_6-tag that can be used to capture the proteins on the sensor chip by chelating to nickel ions. The recombinant proteins were purified as previously reported (7). The media from virus-infected High Five cultures infected at a multiplicity of infection of 10 were harvested by centrifugation at 24, 48, and 72 h post infection and analyzed by PAGE and Western blotting. The concentrations of the recombinant proteins in the supernatants harvested 72 h post infection were determined using an ELISA and rabbit polyclonal serum directed against the hCGβCTP (kindly provided by Dr. Vernon C. Stevens, Ohio State University, Columbus, OH). The concentrations of Bac-hCGβ-WT and Bac-hCGβ-R68E supernatants were 1–2 μg/ml and 2–3 μg/ml, respectively, based on a comparison with hCGβ (Zymed Laboratories, Inc., South San Francisco, CA). To determine whether the β-subunits were aggregated in the supernatant, the nondenatured proteins were analyzed by size exclusion chromatography using the SMART system (Amersham Pharmacia Biotechnology Ltd., Little Chalfont, Buckinghamshire, UK). Baculovirus supernatants (50 μl) were separated on a Sepharose 12 PC 3.2/30 column (bed volume of 2.3 ml) (Amersham Pharmacia Biotechnology Ltd.) at a speed of 50 μl/min. Twenty fractions of 50 μl were collected, and 10% SDS-PAGE and Western blotting using an hCGβCTP-specific rabbit polyclonal serum were used to determine the presence of recombinant protein in the fractions. The molecular weight of the hCGβ subunits was assessed using a High and a Low Molecular Weight Calibration kit (Amersham Pharmacia Biotechnology Ltd.) containing catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa).

Antibodies

The INN-2 mAb (β1), INN-22 mAb (β2), INN-58 mAb (β5), INN-64 mAb (β6), and INN-68 mAb (β7) (12) were HPLC-purified IgG1. Fab fragments of the mAbs were generated using the Pierce Immunopure Fab kit following the manufacturer's instructions (Pierce Chemical Co., Cheshire, UK). The mouse IgGs were digested by immobilized papain for 5 h before the Fc fragment, together with undigested Igs, was removed by passage over a Protein A column. The composition of the Fab fractions was examined using SMART size exclusion chromatography as described above, PAGE, and Western blotting. The concentration of each Fab/antibody solution was determined using the Bradford protein assay following the manufacturer's instructions (Bradford assay protein quantification kit; Pierce Chemical Co.).

Surface Plasmon Resonance

Antigen/antibody complex formation was recorded using a Biacore X biosensor (Biacore, Uppsala, Sweden) and analyzed using the BIAevaluation 2.1 software. All solutions were prepared in HEPES-buffered saline (HBS) at pH 7.5 and passed through the flow cell at a speed of 20 μl/min. The His_6-tagged recombinant hCGβ proteins, as well as all the Fab fragments, were extensively dialyzed against HBS, pH 7.5, before use. Each binding cycle was composed of four events. First, the nitrilotriacetic acid chip was cleaned using 20 μl of 10% sodium dodecyl sulfate followed by 20 μl of 0.1M NaOH. Second, 20 μl of 25 mM NiCl was passed through the flow cell, resulting in an increase of 80 resonance units (RU) due to the noncovalent binding of the nickel ions. Third, 50 μl of the dialyzed baculovirus-derived protein was injected at a concentration selected to give an increase of 500 RU. Finally, 50 μl of a solution containing the appropriately diluted Fab fragments was injected into the flow cell. Once the antibody had passed the surface, the formed complex was washed with HBS at pH 7.5 for an additional 1000 sec to dissociate bound antibody. A minimum of 15 sensorgrams was used for the determination of the association and dissociation constants of each antigen/Fab pair. To obtain an accurate calculation of the kinetic constants, triplicate sensorgrams of five different concentrations for each Fab fragment were obtained. INN-2 was used at concentrations varying between 8.76×10^{-7} M and 8.76×10^{-8} M; INN-22 was used at range between 11.67×10^{-7} M and 1.167×10^{-7} M; the concentrations of INN-58 Fab varied between 19×10^{-7} M and 1.58×10^{-7} M; only five concentrations of INN-64 Fab (8.84×10^{-7} M to 1.77×10^{-7} M) were necessary to establish the association and dissociation rates for the binding of this antibody to Bac-hCGβ-WT; and INN-68 was used at concentrations ranging between 11.2×10^{-7} M and 2.24×10^{-7} M. All experiments were performed at 25 C. Both the association and dissociation curves were recorded. Before

each analysis, the slow release of the His_6-tagged protein was recorded and subtracted from the sensorgrams showing the binding characteristics of the antibodies. The sensorgrams were finally normalized and overlaid before analysis in a 1:1 Langmuir model using the BIAevaluation 2.1 program. This program uses nonlinear regression analysis for the determination of rate binding constants for macromolecular interactions.

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REFERENCES

1. Hearn JP, Gidley-Baird AA, Hodges JK, Summers PM, Webley GE 1988 Embryonic signals during the periimplantation period in primates. J Reprod Fertil Suppl 36:49–58
2. Talwar GP, Singh O, Pal R, Chatterjee M, Sahai P, Dhall K, Kaur J, Das SK, Suri S, Buckshee K, Saraya L, Saxena BN 1994 A vaccine that prevents pregnancy in women. Proc Natl Acad Sci USA 91:8532–8536
3. Talwar GP, Singh OM, Gupta SK, Hasnain SE, Pal R, Majumbar SS, Vrati S, Mukhopadhay A, Srinivasan J, Deshmukh U, Gonga S, Mandokhot A, Gupta A 1997 The HSD-hCG vaccine prevents pregnancy in women: feasibility study of a reversible safe contraceptive vaccine. Am J Reprod Immunol 37:153–161
4. Talwar GP 1999 Vaccines and passive immunological approaches for the control of fertility and hormone-dependent cancers. Immunol Rev 171:173–192
5. Stevens VC 1999 Antifertility vaccine. In: Perlmann P, Wigzell H, eds. Handbook of experimental pharmacology. New York: Springer-Verlag; 443–461
6. 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 β subunit. J Reprod Immunol 31: 21–36
7. Chiesa MD, Martensen PM, Simmons C, Porakishvili N, Justesen J, Dougan G, Roitt IM, Delves PJ, Lund T 2001 Refocusing of B-cell responses following a single amino acid substitution in an antigen. Immunology 103:172–178
8. Porakishvili N, Chiesa MD, Chikadze N, Martenson 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
9. 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
10. Laylor R, Porakishvili N, De Souza JB, Playfair JH, Delves PJ, Lund T 1999 DNA vaccination favours memory rather than effector B cell responses. Clin Exp Immunol 117: 106–112
11. Berger P, Panmoung W, Khaschabi D, Mayregger B, Wick G 1988 Antigenic features of human follicle stimulating hormone delineated by monoclonal antibodies and construction of an immunoradiometric assay. Endocrinology 123:2351–2359
12. Berger P, Klieber R, Panmoung W, Madersbacher S, Wolf H, Wick G 1990 Monoclonal antibodies against the free subunits of human chorionic gonadotropin. J Endocrinol 125:301–309
13. 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 gonadotropin. J Mol Endocrinol 32:571–581
14. Berger P, Kofler R, Wick G 1984 Monoclonal antibodies against human chorionic gonadotropin (hCG). II. Affinity and ability to neutralize the biological activity of hCG. Am J Reprod Immunol 5:157–160
15. 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 Biol 23: 1–38
16. Lund T, Delves PJ 1998 Immunological analysis of epitopes on hCG. Rev Reprod 3:71–76
17. Ross TM, Xu Y, Bright RA, Robinson HL 2000 C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge. Nat Immunol 1:127–131
18. Mathiesen I 1999 Electropermeabilisation of skeletal muscle enhances gene transfer in vivo. Gene Ther 6:508–514