Investigation of factors influencing the immunogenicity of hCG as a potential cancer vaccine

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Keywords

Human chorionic gonadotropin; cancer vaccine; Hsp70; adjuvant; B cell response

Abbreviations

CTP, C-terminal peptide; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride; GDA, glutaraldehyde; Hsp70, heat shock protein 70; hCG, human chorionic gonadotropin; KLH, keyhole limpet haemocyanin; LH, luteinizing hormone; Ova, ovalbumin.

Summary

Human hCG and its β-subunit (hCGβ) are tumour autocrine growth factors whose presence in the serum of cancer patients has been linked to poorer prognosis. Previous studies have shown that vaccines, which target these molecules and/or the 37 amino acid C-terminal hCGβ peptide (hCGβCTP), induce antibody responses in a majority of human recipients. Here we explored whether the immunogenicity of vaccines containing an hCGβ mutant (hCGβR68E, designed to eliminate cross-reactivity with luteinizing hormone) or hCGβCTP could be enhanced by coupling the immunogen to different carriers (KLH or Hsp70) using different cross-linkers (EDC or GAD) and formulated with different adjuvants (RIBI or Montanide ISA720).

While there was little to choose between KLH and Hsp70 as carriers, their influence on the effectiveness of a vaccine containing the BAChCGβR68E mutant was less marked, presumably because being a foreign species, this mutant protein itself might provide T-helper epitopes. The mutant provided a significantly better vaccine than the hCGβCTP peptide irrespective of the carrier used, how it was cross-linked to the carrier or which adjuvant was used when hCG was the target. Nonetheless, for use in humans where hCG is a tolerated self-protein, the need for a carrier is of fundamental importance. Highest antibody titres were obtained by linking the BAChCGβR68E to Hsp70 as a carrier by GAD and using RIBI as the adjuvant, which also resulted in antibodies with significantly higher affinity than those elicited by hCGβCTP peptide vaccine. This makes this mutant vaccine a promising candidate for therapeutic studies in hCGβ-positive cancer patients.

Introduction

The pregnancy hormone human chorionic gonadotrophin (hCG) is a member of the glycoprotein hormone family. Like the other members of this family, luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH), hCG is a heterodimeric molecule consisting of a common α-chain non-covalently associated with a hormone-specific β-chain. Initially, hCG is expressed in the early embryo and is required for implantation into the uterus [1]. Subsequently, synthesis shifts to the placental trophoblast where it stimulates the corpus luteum to produce progesterone and estrogen to ensure its maintenance for the duration of the pregnancy. The pioneering studies of Talwar and colleagues have shown that antibody-mediated bioneutralization of hCG in women indeed prevents pregnancy [2, 3].

Highly sensitive assays have identified very low levels of hCG or hCGβ expression in normal tissues of both men and non-pregnant women, but the function of these hormones in this context has still to be elucidated [4]. hCG is also a biomarker for the detection of patients with placental and trophoblast-derived cancers and patients with germ-cell derived tumours. Importantly, the hormone-specific β-subunit hCGβ has been associated with a wide range of epithelial tumours ranging from bladder, lung, oral/facial, breast, cervical, ovarian, vaginal, prostate, renal and pancreatic carcinomas [5-7]. Although the full biological role of hCGβ in these cancers is still being elucidated, model systems have shown that hCGβ is necessary for survival of the bladder cancer SCaBER [7] and the cervical cancer HeLa [8] cell lines In these systems hCGβ may be functioning as an anti-apoptotic growth factor [8-10]. Furthermore, high titres of hCG-specific antibody prevented the growth of an hCGβ-expressing hepatoma H22 cell line xeno-grafted into mice [11]. This latter study showed also that the induced anti-hCGβ antibodies significantly reduced angiogenesis in the H22 grafts. There is also evidence to implicate hCGβ in metastasis and invasion of cancer cells through

down regulation of E-cadherin [12], which normally prevents invasiveness of carcinoma cells [13]. In 2010, a review of the 43 papers that listed hCGβ as a cancer biomarker, identified 20 (47%) where the expression of hCGβ was associated with poor prognosis and accelerated death [6]. It would seem logical therefore, that bioneutralization of hCGβ in these cancers could improve the survival of cancer patients thus identifying the subunit as an important target for anti-cancer therapy.

hCG is a structurally and immunologically well-characterized molecule. The crystal structure has shown it to be a member of the cysteine-knot superfamily of growth factors [14,15]. The use of competitive immunoassays [16-21] and amino acid substitutions [22,23] have identified sixteen immunological regions on hCG of which five epitopes have been mapped on the α-subunit, seven identified on the β-subunit and four epitope clusters located on the interface between the α- and β-chains. The hormone specific β-subunit of hLH shares 85 % of the amino acid sequence with the first 110 amino acid residues in hCGβ, which accounts for the dominant immune epitopes on hCG being shared with LH so that hCG-induced antibodies may cross-react with LH [23]; it is also likely that many of the T-cell epitopes will be shared with LH.

With the aim of neutralizing the role of hCG in pregnancy, Talwar and his group developed a heterospecies hCG anti-fertility vaccine consisting of an ovine α- and human β-subunit conjugated to tetanus or diphtheria toxoid. In a ground-breaking human phase II trial with this vaccine they found only one pregnancy in 1224 cycles in the immunized women who produced anti-hCG antibodies levels above 50 ng/ml [3]. The effect of the vaccine was reversible because pregnancies were detected whenever the hCG antibody levels fell below the protective threshold [2,3]. Although, to our knowledge, the use of this vaccine has not been pursued further, it nevertheless demonstrates that it is possible to develop bioneutralizing hCG vaccines in humans and indeed, in the last two decades, hCGβ has been

examined as a target for anti-cancer vaccines. However, the heterospecies vaccine protected only 80% of the immunized women in this trial who produced the bioneutralizing levels of anti-hCG antibodies. The need for enhanced immunogenicity was recognized by Talwar, leading to his development of new formulations including the use of *E.coli* endotoxin and killed mycobacteria to boost the immune response [24]. We have previously shown that immunization with a GnRH analogue conjugated to mycobacterial hsp70 as a carrier reduced the fertility of male mice [25]

Stevens promoted the use of the 37 amino acid C-terminal segment of hCGβ (hCGβCTP), not present in LHβ, as a possible hCG-specific vaccine candidate [26]. Indeed, in a human phase I trial involving 37 patients with recurrent or metastatic tumours, Triozzi *et al* showed that synthetic hCGβCTP covalently attached to diphtheria toxoid induced hCG-specific antibodies at levels between 0.1-2 μg IgG per ml (1-20 nM) in a dose-dependent manner [27,28]. The effect of the vaccine on the tumours was not evaluated although Triozzi *et al* noted that two patients with colorectal cancers showed tumour regression [28]. They did not, however, assess whether this was due to the induced hCG-binding antibodies or because of their observation that the carrier and adjuvant induced strong Th1 as well as Th2 cytokine responses in all patients. However, we consider that the C-terminal segment is not an ideal immunological target for two reasons. First, it contains four 0-linked glycosylation sites, which are occupied in native hCG and its free hCGβ subunit so that the carbohydrate chains could physically block or mask some of the potential B-cell epitopes in the C-terminal segment. Secondly, it is a highly flexible molecule with no fixed structure and is thus entropy-rich, making it a poor immunogen favoring the production of antibodies with low affinity for the hCGβ target. In another approach to overcome the poor immunogenicity of hCGβCTP, Xiangbing *et al*. constructed a fusion protein consisting of heat shock protein (Hsp65) with ten tandem repeats of hCGβ109-118 and a copy of

hCGβ109-145 peptide. This vaccine was able to suppress the growth of mouse hepatoma H22 cells in mice [11], but it remains to be seen whether it will be able to induce bioneutralizing responses in an outbred populations like humans with their diverse HLA haplotypes.

We have previously reported an alternative hCG vaccine candidate consisting of hCGβ with a single amino residue substitution (R68E) and which has minimal LH-cross-reactivity [23,29,31]. We showed that the entropy-rich C-terminal segment becomes electrostatically fixed through the interaction between the Glu68 residue and the lysine and arginine residues in the C-terminal segments. This directs the immune response towards hCGβ-specific epitopes, including those in the C-terminus of the β-subunit, in both rabbits and mice using both conventional protein as well as DNA immunization [23, 29-31].

The present study evaluates whether our mutant is a more potent hCG-specific vaccine candidate than hCGβCTP, whilst at the same time addressing the concerns regarding LH-cross reactivity raised in the Talwar hCG trials [3]. We have also sought to improve the immunogenicity of hCGβCTP and hCGβR68E by covalent coupling to either Hsp70 or keyhole limpet haemocyanin (KLH) and evaluated two oil-in-water adjuvant systems RIBI and Montanide ISA72. We report here that our mutant hCGβR68E is superior to CTP as a vaccine candidate.

Material and Methods

Reagents

Recombinant hCGβ produced in CHO cells was purchased from Sigma-Aldrich (UK); Recombinant BAChCGβR68E was purified from baculovirus-infected HiFive insect cells (see below). The C-terminal peptide (hCGβCTP) representing the amino acid residues 108-145 of hCGβ was synthesized *in vitro* and kindly provided by Professor Vernon C Stevens, Ohio State University, Columbus, OH or synthesized in house. Recombinant endotoxin-free Hsp70 was a gift from Professor Theo Verrips, Utrecht University, and KLH was purchased from Sigma-Aldrich (IL). The CTP-specific mAbs used in the study were OT3A2 (kindly provided by Dr E Bos, NV Organon, Oss, The Netherlands) and 2F4/3 (Sigma-Aldrich). The carrier –specific antibodies used were rabbit anti-KLH IgG (Sigma-Aldrich) and rabbit anti-Hsp70 antiserum kindly provided by Professor Theo Verrips.

Production and purification of baculovirus-produced hCGβ-R68E

The pBAC2hCGβR68E baculovirus expression plasmid for production of recombinant hCGβR68E with a C-terminal His_6-tag [29] was transiently introduced into HiFive insect cells (InVitrogen) and a single recombinant virus expressing BAChCGβR68E was isolated and expanded. For large scale production of the recombinant protein the insect cells were grown in roller flasks in Express Five medium ((InVitrogen) supplemented with 1% penicillin/streptomycin and 16 mM L-glutamine to a density of $1.5x10^5$ cells per ml at 28^oC. The cells were infected with the recombinant baculovirus using a multiplicity of infection (MOI) of 10 and the supernatant harvested 72 hr post infection, centrifuged and immediately stored at -70^oC. Recombinant BAChCGβR68E was affinity purified in batches

of 50-200 ml insect cell supernatants after dilution with an equal volume of 20 mM Na_2HPO_4, 0.5 M NaCl pH 7.3 containing protease inhibitors (Sigma-Aldrich). The BAChCGβR68E was then centrifuged at 5000 rpm, filtered through a 0.45 μ filter, and loaded onto HiTrap columns according to the manufactures' instructions (GE Healthcare Life Sciences) using a HPLC system using a flow rate of 1 ml/min. After extensive washing with 20 mM Na_2HPO_4, 0.5 M NaCl pH 7.3 followed by 20 mM Na_2HPO_4, 0.5 M NaCl, 25 mM imidazole pH 7.3, the recombinant protein was eluted with 20 mM Na_2HPO_4, 0.5 M NaCl, 400 mM imidazole pH 7.3 and concentrated to 0.65-1.0 ml using Centricon YM-10 columns (Millipore) centrifuged at 5000 rpm. One μl of the initial supernatant and purified samples were separated on a 12.5% SDS-PAGE using Phast System (GE Healthcare Life Sciences), silver stained and transferred to nitrocellulose membrane. Immunoblot analysis was carried out using hCGβ-specific monoclonal antibodies and a 3,3'-diaminobenzidine (DAB) enhanced liquid substrate System tetrahydrochloride for chromogenic detection (Sigma).

Coupling of CTP and hCGβ-R68E to carrier proteins

The recombinant proteins were conjugated to Hsp70 and KLH using either glutaraldehyde (GAD) (Sigma-Aldrich UK) or 1-ethyl-3(3-dimethylaminopropyl)carboiimide (EDC) (Pierce UK) using a two-step coupling procedure. For coupling with GAD, synthetic hCGβCTP (0.75 mg) or BAChCGβR68E (1 mg) was incubated with 0.075% GAD for 2 hr at 4^oC with gentle rotation followed by desalting using a PD10 column (Pharmacia). For cross-linking with EDC, the two-step protocol recommended by the manufacturer was followed. In brief, hCGβCTP (0.75 mg) or hCGβ-R68E (1 mg) was dialyzed into 0.1M 2-(N-morpholino)ethanesulphonic acid (MES), 0.5M NaCl, pH 6.0, incubated with 2 mg of EDC

for 15 min at RT, de-salted using a PD10 column and then added to an equal volume of Hsp70 or KLH in PBS and incubated at room temperature (RT) for 2 hr with gentle rotation.

The success of conjugation to Hsp70 was examined using analytical HPLC gel filtration, SDS gel electrophoresis and Western blotting using PhastSystem and highly sensitive sandwich ELISA using antibodies to the carriers and monoclonal CTP-specific OT3A2 mAb which recognises the amino acids 133-139. The molar coupling efficiency (number of antigen molecules per mole of carrier) was estimated by determining the amino acid composition of the final Hsp70-hCGβCTP and Hsp70-BAChCGβR68E conjugates and calculating the molar concentration of the antigens using selected amino acid residues. The KLH-conjugate was too large for this analysis.

Immunisation of mice

Six-week old female BALB/c mice (Harlan Olac, Bicester UK) were kept according to UK Home Office guidelines and the experimental procedures were covered by Home Office Animal Project guidelines. The animals used were primed with a 10 μg aliquot of the Hsp70- or KLH-conjugate containing hCGβCTP or BAChCGβR68E in RIBI (Sigma-Aldrich UK) or Montanide ISA720 (SEPPIC, Paris, France) followed by a boost 21 days later. Two weeks after the boost, the animals were exsanguinated and the serum antibodies titered using direct binding ELISA. For this, Nunc MaxisorpC 96 well flat-bottomed microtitre plates were coated at 4^{o}C overnight with 50 μl recombinant hCGβ (Sigma-Aldrich UK), hCG, or ovalbumin at 1 μg/ml or hCGβCTP peptide at 5 μg/ml in 50 mM carbonate-bicarbonate buffer (CBB) pH 9.6. After washing the plates extensively with PBS they were blocked with 2% w/v bovine serum albumin (BSA) in PBS for 30 min at RT followed by washes with PBS. The sera were serially diluted in PBS, 0.05% Tween 20, 1% BSA and 50 μl was added to

each well and incubated for 2 h at 37^{o}C. The plates were washed extensively with PBS, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or subclass-specific IgG (Sigma-Aldrich UK) for 1 h at 37^{o}C, washed and developed with 50 µl tetramethylbenzidine (TMB) and read at A_{630} using an ELISA plate reader. The avidity was determined using ELISA essentially as described above using antiserum at a concentration of 80% of the plateau binding followed by an incubation of the antibody-antigen complexes with increasing concentration of (0.031 M – 8M) ammonium thiocyanate for 15 min at RT [33]. The plates were subsequently washes and developed using horseradish peroxidase-conjugated goat anti-mouse IgG as described above. 50% inhibition of the ammonium thiocyanate concentration was determined as the avidity index.

Statistical analysis

A 10 point standard curve of antiserum dilution against signal (absorbance) was constructed for each antiserum produced from each mouse using a 4 parameter Logistic Curve Fitting (elisa analysis.com). The highest dilution that could be distinguished from the blank (mean absorbance + 2SD from ovalbumin-immunized mice) was recorded as an index of immunogenic vaccine potency. The independent effects of different carriers, linkers and adjuvants on the titre were analyzed using general linear model multivariate analysis of variance with a hierarchical design and Tukey's HSD post hoc analysis. A student t-test was used for the isotype and avidity analysis.

Results

Characterization of conjugates

Affinity-purified BAChCGβR68E with a molecular weight of 25 kDa is smaller than the 45 kDa CHO-produced hCGβ (Figure 1A) due to differences in the structural complexity of the carbohydrate chains but not the diminished degree of glycosylation [34]. As reported previously, baculovirus-produced recombinant wild-type and mutant hCGβ subunit fold correctly as judged by their full recognition of a panel of conformation-dependent monoclonal antibodies [30]. Once purified, BAChCGβR68E as well as synthetic hCGβCTP were chemically coupled to Hsp70 and to KLH using GAD, which we had used previously to chemically attach GnRH to Hsp70 [25], as well as the zero-length crosslinker, EDC. Western blot analysis of the Hsp70-based conjugates shows covalent attachment of the immunogens to the carrier (Figure 1B). We estimated the relative molar conjugation ratio of hCGβR68E:Hsp70 and hCGβCTP:Hsp70 as 4.7:1 and 31:1, respectively, by determining the increase in the molar content of tyrosine and valine, respectively, in a full amino acid quantification of conjugates relative to the native Hsp70 (Figure 1C). It was not possible to get a meaningful estimate of the coupling efficiency of BAChCGβR68E and hCGβCTP to KLH due to its very large molecular weight of $7.8x10^3$ kDa.

Immunogenicity of hCBβR68E versus hCGβCTP

The immunogen-carrier complexes were used to immunize groups of female BALB/c mice with two different oil-in-water adjuvants, RIBI and Montanide ISA720, chosen because they have both been approved for human use (Table 1). The specificity of the elicited antibodies was characterized using endpoint titration ELISAs against the target antigens hCG,

hCGβ and hCGβCTP and using ovalbumin as the negative control. The dilutions representing the highest dilution that could be distinguished from the mean absorbance plus 2SD of ovalbumin were recorded (Figure 2). There was no significant difference between the results obtained with intact hCG and with the **recombinant hCGβ** when used as target antigens. We therefore combined the results obtained with these two antigens in our statistical analysis. Given that both BAChCGβR68E and hCGβCTP produced immune-responses that are likely to be effectively devoid of LH crossreactivity, our first question was: which is the better immunogen? When targeting hCG/hCGβ, the BAChCGβR68E antisera showed better binding to the antigens than the antisera elicited with hCGβCTP (for BAChCGβR68E mean titre was 1:26500; for hCGβCTP mean titre was 1:12600, $p<0.0001$) (Table 2, Figure 3). Even when titered against the synthetic hCGβCTP peptide as the target antigen, we found that the baculovirus-derived recombinant protein elicited more potent immune response than that observed with hCGβCTP conjugates as immunogens (BAChCGβR68E mean titre 1 in 210800, hCGβCTP mean titre 1 in 54500, $p=0.039$; Figure 4, Table 2).

Enhancing the immunogenicity

In the reported phase II trial with the hetero-CG vaccine, a substantial fraction (~20%) of the immunized women failed to develop protective immunity [24]. One likely explanation could be that the vaccine-formulation used was suboptimal for this group of recipients for genetic and/or immunological reasons. It is therefore possible that the number of poor responders could be reduced by using a vaccine with greater immunological potency. We decided therefore to explore the effect of different immunological carriers, chemical linkers and adjuvant systems on immunogenicity. No statistical differences were observed between Hsp70 and KLH as carriers irrespective of the immunogen (BAChCGβR68E versus hCGβCTP) or linker (GAD versus EDC) (Figure 3).

When considering the adjuvant system (RIBI versus Montanide ISA720) the BAChCGβR68E immunogen elicited no statistical difference in the antibody titres irrespective of the adjuvant, linkers or antigen targets used with the one exception of RIBI being the superior adjuvant with the hCGβCTP target (Figure 3). However, the CTP-vaccine did reveal differences. When titered on its biological target hCG/hCGβ, the CTP-immunogen formulated with RIBI produced significantly higher titre antibodies than those obtained using the Montanide ISA720-formulation but only when the synthetic peptide had been crosslinked to its carrier with GAD (mean titre RIBI 1:15000, Montanide ISA720 1:2000, t-test $p<0.05$). When titered against the synthetic hCGβCTP peptide itself, the antisera generated with RIBI elicited significantly higher antibody responses than immunogen adjuvanted with Montanide ISA720 irrespective of the linker ($p<0.001$) (Figure 3).

Combining all the antibody responses to the mutant recombinant BAChCGβR68E revealed no differences in the overall potency of the two adjuvants with respect to affinity (Figure 5A). However, as shown in Figure 5B and 5C the BAChCGβR68E-elicit antibodies binding to hCGβCTP with lower avidity than to hCG but with the same avidity as hCGβCTP-induced antibodies independent of the carrier. For the analysis we used an avidity index defined as the concentration of ammonium thiocyanate required to dissociate 50% of the antigen-antibody complexes as indicated in Figure 5B. Using this index it can be seen that there were no statistical differences in the affinity of the specific antibodies produced by the hCGβCTP immunogens and independent of the carrier when binding to hCGβCTP. In contrast, hCGβCTP-specific antibodies from hCGβCTP-HSP70 immunised mice bound to the synthetic peptide with significant lower affinities (p,0.007) (Figure 5C). We have previously shown that the amino acid substitution in BAChCGβR68E fixed the C-terminal part of hCGβ through electrostatic interaction this masking the immunodominant LH-cross-reactive epitope on hCGβ but enhances a hCGβCTP-specific epitope [29-31]. It is therefore

not surprising that the avidity of the hCGβCTP-specific antibodies were comparable to that induced by hCGβCTP-immunogens and higher than hCGβCTP antibodies induced by hCGβ □□□□□□□□□. In addition, the antibodies induced with at the BAChCGβR68E immunogen had a significantly greater affinity overall than antibodies induced by hCGβ. Surprisingly IgG2a and 2b titres were significantly lower with Montanide ISA720 than with the RIBI formulations (Figure 6).

Our results revealed a clear difference between the immunogens. Collectively, the BAChCGβR68E vaccine formulation gave significantly greater responses against both targets than did the hCGβCTP-based vaccines (Figure 3). Using hCG as target, the hCGβCTP linked to carrier by GAD with RIBI adjuvant gave the best antibody response of the hCGβCTP immunogen group but this was significantly lower than the corresponding result with the BAChCGβR68E mutant ($p<0.014$).

Discussion

Human CG has traditionally been associated with pregnancy, but the recent decades have revealed that hCG and hCGβ are also biomarkers for trophoblastic and epithelial cancers and the presence of hCGβ is predictive for poor survival of patients (recently reviewed in [6]) possibly because it prevents apoptosis or functions as a cancer growth factor. Phase I/II trials of an antifertility vaccine, based on a heterodimeric CG molecule, by Talwar and his group showed that it is possible to break immunological tolerance to hCGβ and thereby elicit sufficient levels of antibodies to prevent pregnancy in immunized women [3]. hCGβ has therefore subsequently been considered as a potential immunotherapeutic anti-cancer vaccine candidate [10,11].

Morse *et al.* and Celldex Therapeutics Inc have recently explored an hCGβ-targeting bladder carcinoma vaccine with a formulation that induced T- as well as B-cell mediated immune responses. It consisted of a fusion protein where the human monoclonal anti-mannose receptor antibody B11 was extended with hCGβ at the C-terminus (CDX-1307). In a phase II trial, CDX-1307 was given with GM-CSF and Toll-like receptor 3 and 7/8 agonists known to enhance the adaptive immune response as well as Cisplatin and Gemcitabine for broader cancer cell targeting [32, 35]. The phase II trial was discontinued after fourteen months due to difficulties in recruiting sufficient number of patients (RK Iles, personal communication). We have argued here that an hCGβ–based vaccine will produce predominantly LH-cross reactive antibodies due to the immune dominance of the shared epitopes. We presume furthermore that the 85% sequence homology between the hormone-specific subunit of LH and the first 110 amino acids of hCGβ indicates that the two hormones also share most of the MHC class I epitopes. Although it is possible that such LH cross-reactivity in both arms of the adaptive immune system may not be of immediate concern for cancer patients, we do argue for hCGβ-specific vaccines that predominantly target the

antibody-mediated arm of the immune system to avoid undesirable long-term complications. Most efforts have been focused on the unique C-terminal peptide of hCGβ. AVI BioPharma Inc has taken a vaccine consisting of hCGβCTP$_{37}$ coupled to diphtheria toxoid (CTP37-DT) through phase I with patients with a number of different epithelial cancers followed by a phase II trials in 77 patients of metastisizing colorectal carcinomas. However, the vaccine-induced hCGβCTP antibodies were not able to neutralize the tumour-derived hCGβ either due to the high entropy of C-terminal segment or because the hCGβCTP antibodies were of low affinity. It is therefore not clear whether the effect in the high responders was related to induction of hCG-specific antibodies or to general stimulation of the immune system by the DT carrier, which elicited a systemic cytokine response [27]. It is, furthermore, possible that a better protection could be achieved in patients with hCGβ-producing cancers.

We show here that our hCGβR68E mutant may be a more suitable immunogen than either hCGβ or hCGβCTP. The Glu68 mutation fixes the CTP via salt bridges to its positive amino acids thereby not only blocking the immunodominant LH cross-reactive epitopes but creating also a novel dominant CTP B-cell epitope possibly located at the novel loop and including the amino acid residues 105-120 [28-30]. BAChCGβR68E conjugated to either Hsp70 or KLH produced significantly higher levels of immune reactive hCG antibodies than hCGβCTP-Hsp70 or hCGβCTP-KLH irrespective whether they were titered against hCG, hCGβ or CTP. However, the difference in the antibody levels was not as pronounced when tittered against CTP. There may be several reasons for this. The molar level of CTP per Hsp70 molecule was 6.6 times higher than for BAChCGβR68E per Hsp70. In addition, the CTP, was a synthetic peptide with at least four known B-cell epitopes some of which may be masked by the four 0-linked carbohydrate residues present in the C-terminal part of BAChCGβR68E. One would therefore expect that not all of the antibody specificities elicited with hCGβCTP formulation, would recognize hCG/hCGβ.

As with the anti-fertility trial of Talwar and colleagues [3], the CTP37-DT vaccine identified a significant group of non-responders [27]. The molecular basis for the inability of 20% of the individuals participating in two trials who failed to respond to the vaccines remains to be elucidated. It is possible that there are genetic reasons for this since the two trials included diverse ethnic patients. However, all patients included in the two trials responded normally to the carrier, demonstrating a functional immune response. Since Moulton *et al.* reported that detectable levels of anti-hCG antibodies were only seen after the second boosting [27], it is possible that enhancing the immunogenicity of the immunogen or vaccine formulation or repeated boosting may reduce the number of non-responders. We explored whether we could enhance the immunogenicity of BAChCGβR68E or hCGβCTP by coupling the vaccine candidate to different carriers, using different cross-linkers or formulating them with different adjuvants. Whilst these different constructs induced a modest but statistically significant increase in the immunogenicity of hCGβCTP, these improvements were less pronounced with BAChCGβR68E. Nonetheless, even by enhancing the immunogenicity, the hCGβCTP vaccine formulation was not as potent as our mutant molecule. Differences in ability of the antisera to neutralize circulating hCG may be even greater if, as we expect, the high entropy unconstrained CTP immunogen produces a low affinity response. While as mentioned, the effect of conjugation with carrier was relatively modest, perhaps because hCG is a foreign molecule for mice, for human use the involvement of carrier protein would be essential since hCG is a tolerated self-protein. Although the 2 adjuvants did not induce antibodies with overall differences in avidity as revealed by ammonium thiocyanate dissociation, the superiority of RIBI with respect to IgG subclass response and induction of the highest antibody titres emphasize the need for careful attention that needs to be paid to the choice of adjuvant for a vaccine intended for human use. The avidity analysis revealed that when tested on hCGβ CTP peptide-coated plates the antibodies elicited by hCGβCTP and BAChCGβR68E immunogens had the same avidity, which was

significant ($p<0.007$ in student t-test) than hCGβCTP-specific antibodies induced by hCGβ conjugated to the same carrier. However, the affinities of the antibodies produced in hCGβCTP-immunized mice were significantly lower when assayed on plates coated with hCG. This reflects probably more an assay artifact because coating of the CTP peptide will anchor it in a fixed low-entropy conformation. When hCG is coated to the plastic of the 96 well plates the CTP will not all be immobilized the plastic thus having no fixed conformation and be very entropy rich which will reduce the availability of the right binding conformation for the induced antibodies. In addition, maybe the molar concentration of hCGβCTP peptide is higher in the peptide-coated plates. What the avidity data clearly demonstrated is that the avidity of hCG-specific antibodies produced by our mutant immunogen were significantly higher than the antigen-specific antibodies produced by either hCGβCTP or hCGβ immunogens. This makes BAChCGβR68E a much better vaccine candidate.

In conclusion, we have compared two hCGβ-specific vaccine candidates hCGβCTP and BAChCGβR68E delivered using different formulations and report here that the hCGβ mutant BAChCGβR68E is a significantly more potent (or effective) vaccine than hCGβCTP irrespective of the carrier used, how it was crosslinked to the carrier or which adjuvant system used. Highest antibody titres were obtained by linking the BAChCGβR68E to Hsp70 as a carrier by GAD and using RIBI as the adjuvant, and although we do not know whether it will be a superior vaccine that can reduce the fraction of non-responders identified in the phase II trials of hCG vaccines so far, the increased immunogenicity relative to hCGβCTP looks promising.

Figure Legends

Figure 1. Purification of BAChCGβR68E and coupling of the immunogens to carrier proteins. (**A**) 1 μl of affinity purified recombinant BAChCGβR68E was separated by 12% SDS PAGE followed by Western blotting and compared to CHO-produced hCGβ: Lanes 1-3 show rhCGβ at 0.25, 0.5 and 1.0 mg/ml and Lane 4-5 show two batch batches of purified BAChCGβR68E preparations. (**B**) Western blot analysis using the OT3A2 mAb showing the coupling of hCGβCTP and BAChCGβR68E to Hsp70; Lane 1 hCGβCTP; Lane 2 Hsp70; Lane 3 hCGβCTP-Hsp70 conjugated with EDC; Lane 4 hCGβCTP mixed with Hsp70; Lane 5 hCGβCTP; Lane 6 hCGβCTP-Hsp70 conjugated with GAD; Lane 7 BAChCGβR68E-Hsp70 conjugated with EDC, and Lane 8 BAChCGβR68E-Hsp70 conjugated with GAD. (**C**) Evaluation of ratio of hCGβCTP:Hsp70 and BAChCGβR68E:Hsp70 calculated from total amino acid quantification of the conjugates and Hsp70.

Figure 2 Titration of mouse immune sera. The sera from mice immunized with either hCGβCTP- or BAChCGβR68E-conjugate were endpoint titered using direct ELISAs on plates coated with hCG (to the left) and the synthetic hCGβCTP peptide (to the right). The graphs used data that includes both linkers, both carriers and both adjuvants. They show the mean absorbance and ±2SD indicated as bars through each data point. The non-specific binding of the sera was determined using plates coated with ovalbumin (Ova).

Figure 3 Statistical analysis of the endpoint titration of the sera from BALB/c mice immunized with hCGβCTP- or BAChCGβR68E-conjugates titered on hCGβCTP (upper diagram) or hCG/hCGβ (lower diagram). The $\log_{10}$ dilution of the endpoints for the relevant

groups are shown using box-and-whisker diagrams where the median is indicated with a horizontal bar, the 1QR by a box, the whisker represents the range of data and the mean and 2SD of the log transformed data. Dilution end points were defined as the highest dilution that could be distinguished from the blank (mean absorbance + 2SD from ovalbumin-immunized mice). * Denotes significance between BAChCGβR68E immunogen compared to the hCGβCTP immunogen ($p<0.05$). + Indicates the significant difference in titres between the adjuvant RIBI and Montenide ISA720 ($p<0.05$). * Indicates significant differences compared to hCGβCTP immunogen with all other conditions the same.

Figure 4 Statistical analysis of the antibody response to BAChCGβR68E immunogen generated as a free subunit or used when conjugated to Hsp70 or KLH all combined with the adjuvant.

Figure 5 (A) Avidity of sera from the mice immunised with a BAChCGβR68E immunogen using the adjuvant RIBI and Montenide ISA720 adjuvant formulations produce antibodies with as identical ammonium thiocyanate dissociation when tittered on hCGβ (∗$p<0.0021$ and **$p<0.0019$ Student t-test). (B) However, the dissociation of the antigen-antibody complexes in sera from mice immunised with BAChCGβR68E-HSP70 immunogen using the adjuvant RIBI tittered on hCG (squares) and CTP (triangles) was different. We define an avidity index as the concentration of ammonium thiocyanide that results in dissociation of 50% of the antibody-antigen complex (indicated by the stippled lines). (C) The relative avidity indexes represented by 50% inhibitory concentrations of antibodies rose to constructs for CTP (dark) and hCGß (light). Student t-test was used to determine the statistical significance as indicated).

Figure 6 IgG subclass responses in mice immunised with the BAChCGβR68E immunogen using the RIBI and Montenide ISA720 adjuvant formulations and tittered on hCGβ.

Contributions

PJD, TL and IMR conceived this study. NK, NC, JM, JDM, NP, PMM and JJ carried out the experiments. FH and NP performed the statistical analysis. TL, FH and IMR wrote the manuscript. All authors have seen and approved the final version of the manuscript.

Conflict of Interest statement

None. Although Prof. Roitt received generous support from Igeneon, GmBH Austria, the intervening bankruptcy of the company eliminated any possibility of a conflict of interest.

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