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Porakishvili, N., Roitt, I.M., Lund, T., Delves, P.J., Justesen, J., Martensen, P., Chikadze, N. and Kvirkvelia, N.

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NinoKvirkvelia[1§#], Nino Chikadze[2§], Julia Makinde[1#], Jeffrey DMcBride[1#], Nino Porakishvili[3], FrankA Hills[4], Pia M Martensen[5], Just Justesen[5], Peter J Delves[1], Torben Lund[1,3*]& Ivan M Roitt[1,3]

1 Division of Infection and Immunity, University College London, UK

2 I. Javakhishvili State University, Tbilisi,,Georgia

3 Department of Biomedical Sciences, University of Westminster, London, UK

4 Department of Natural Sciences, School of Science and Technology, University of Middlesex, London, UK

5 Department of Biomedical Sciences, University of Westminster, London, UK

6 Departmentof Molecular Biology and Genetics, Aarhus University, Denmark

§ N.K and N.C contributed equally to this work

Present addresses: Department of Medicine, Augusta University, Augusta, GA (N. Kvirkvelia), IAVI Human Immunology Laboratory, Department of Medicine, Imperial College London, London, UK(JuliaMakinda) and Thermo Fisher Scientific, Dartford, UK (JD McBride)

* Corresponding Author: Torben Lund, Department of Natural Sciences, Room H132, School of Sciences and Technology, University of Middlesex, The Burroughs, London NW4 4BT, UK, t.lund@mdx.ac.uk

Keywords

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

Abbreviations

CTP, C-terminal peptide; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimidehydrochloride; 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 tumourautocrine 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/orthe 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 eliminatecross-reactivity with luteinizing hormone) or hCGβCTPcould beenhanced 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 thanthe hCGβCTP peptide irrespective of the carrier used, how it was cross-linked to the carrier or which adjuvant was used when hCGwas 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 byhCGβCTP 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 implantationinto the uterus[1]. Subsequently, synthesis shifts to the placental trophoblastwhere it stimulates the corpus luteum to produce progesterone and estrogen to ensure itsmaintenancefor the duration of the pregnancy.The pioneering studies ofTalwar and colleagues have shown that antibody-mediated bioneutralization of hCG in women indeed prevents pregnancy [2, 3].

Highly sensitive assays haveidentified 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]. hCGis 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 hCGβis necessary for survival of the bladder cancer SCaBER[7] and the cervical cancer HeLa[8]cell linesIn these systems hCGβ may be functioning as an anti-apoptotic growth factor [8-10].Furthermore, high titres of hCG-specific antibody preventedthe growth of an hCGβ-expressing hepatomaH22 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 implicatehCGβ in metastasis and invasion of cancer cells through down regulation of E-cadherin [12], which normally preventsinvasiveness of carcinoma cells [13]. In 2010,a review of the 43 papers that listedhCGβ 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 hCGβin these cancers could improve the survival of cancer patients thus identifying the subunit as animportant target for anti-cancer therapy.

hCGis 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 specificβ-subunit of hLH shares 85 % of the amino acid sequence with the first 110 amino acid residues in hCGβ, which accounts forthe dominant immune epitopes on hCGbeing 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 heterospecieshCG anti-fertility vaccine consisting of an ovine α- and human β-subunit conjugated to tetanus or diphtheria toxoid. In aground-breakinghuman 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 bioneutralizinghCG vaccines in humans and indeed, in the last two decades,hCGβ hasbeen

examined as a target for anti-cancer vaccines. However, the heterospecies vaccine protected only 80% of the immunized women in this trial whoproduced 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 segmentof hCGβ (hCGβCTP), not present in LHβ, as a possible hCG-specific vaccine candidate [26]. Indeed, in a human phase Itrial involving 37 patients with recurrent or metastatic tumours,Triozzi*et al* showed thatsynthetic 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 four0-linked glycosylation sites,which are occupied in native hCG and its free hCGβ subunit so that thecarbohydrate chains couldphysically block or mask some of the potential B-cell epitopes in the C-terminal segment. Secondly, itis a highly flexible moleculewith no fixed structure and is thusentropy-rich,making it a poor immunogenfavoring 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], butit 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 hCGβwith 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 potenthCG-specific vaccine candidate thanhCGβCTP, whilst at the same time addressing the concerns regarding LH-cross reactivity raised in the TalwarhCG trials [3].We have also sought to improve the immunogenicity of hCGβCTP and hCGβR68E by covalent coupling to either Hsp70 orkeyhole limpet haemocyanin (KLH) and evaluated two oil-in-water adjuvant systems RIBI and MontanideISA72. 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 fromProfessor 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^{o}C. 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^{o}C. Recombinant BAChCGβR68E was affinity purified in batches of 50-200 ml insect cell supernatants after dilutionwith an equal volume of 20 mM Na_2HPO_4, 0.5 M

NaClpH 7.3 containing protease inhibitors (Sigma-Aldrich). The BAChCGβR68E was then centrifuged at 5000 rpm, filtered through a 0.45 µ filter, and loaded ontoHiTrap columnsaccording 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 NaClpH 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, 400mM imidazole pH 7.3 and concentrated to 0.65-1.0 ml using Centricon YM-10 columns (Millipore) centrifuged at 5000 rpm. Oneµl 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) or1-ethyl-3(3-dimethylaminopropyl)carboiimide(EDC) (Pierce UK) using a two-step coupling procedure. For coupling with GAD, syntheticCGβCTP (0.75 mg) or BAChCGβR68E (1 mg) was incubated with 0.075% GADfor 2 hr at 4^{o}C 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 conjugationto Hsp70 wasexamined 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 hCGβCTPor 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 antibodiestitered using direct binding ELISA. For this, NuncMaxisorpC 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 and50μl 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 IgGor 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 bindingfollowed 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 (elisaanalysis.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 kDais 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-typeand mutant hCGβ subunitfold correctly as judged by their full recognition of a panel of conformation-dependent monoclonal antibodies [30]. Once purified,BAChCGβR68E as well as synthetic hCGβCTPwere 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 showscovalent 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 conjugatesrelative 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 itsvery 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 MontanideISA720,chosen because they have both been approved for human use (Table 1). The specificity of the elicited antibodies was characterized using endpoint titration ELISAsagainst the target antigens hCG, hCGβ andhCGβCTP 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 wererecorded(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 andhCGβCTP producedimmune-responses that are likely to be effectively devoid of LHcrossreactivity,ourfirst question was: which is the betterimmunogen?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 titeredagainst the synthetic hCGβCTP peptide as the target antigen, we found that the

baculovirus-derived recombinant protein elicited more potent immune response than that observed withhCGβCTP conjugates as immunogens (BAChCGβR68E mean titre 1 in 210800, hCGβCTP mean titre 1 in 54500,p=0.039; Figure4, Table 2).

Enhancing the immunogenicity

In thereported 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 decidedtherefore 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 immunogenelicited 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 onitsbiological target hCG/hCGβ, the CTP-immunogen formulated with RIBI produced significantly higher titre antibodies than those obtained using the Montanide ISA720-formulationbut 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 titeredagainst the synthetic hCGβCTPpeptide itself, the antisera generated with RIBI elicited significantlyhigher antibody responsesthan immunogenadjuvanted with Montanide ISA720 irrespective of the linker ($p<0.001$)(Figure 3).

Combining all the antibody responses to the mutant recombinant BAChCGβR68Erevealed 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 ashCGβCTP-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 hCGβCTPimmunogensand independent of the carrier when binding tohCGβCTP. In contrast,

hCGβCTP-specificantibodies from hCGβCTP-HSP70 immunised mice bound tothe synthetic peptide with significant lower affinities (p,0.007) (Figure 5C). We have previously shown that the amino acid substitution in BAChCGβR68Efixed the C-terminal part of hCGβ through electrostatic interaction this masking the immunodominant LH-cross-reactive epitope on hCGβ but enhances ahCGβCTP-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 BAChCGβR68Eimmunogen 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 BAChCGβR68Evaccine 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 hCGβCTPimmunogen group but this was significantly lower than the corresponding result with the BAChCGβR68Emutant ($p<0.014$).

Discussion

Human CG has traditionally been associated with pregnancy, but the recent decades have revealed that hCG and hCGβare also biomarkers for trophoblastic and epithelial cancers and the presence of hCGβis predictive for poor survival ofpatients(recently reviewed in[6]) possibly because itprevents 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 tohCGβand thereby elicit sufficient levels of antibodies to prevent pregnancy in immunized women[3].hCGβhas therefore subsequently been considered as a potentialimmunotherapeutic anti-cancer vaccine candidate [10,11].

Morse *et al.*and Celldex Therapeutics Inchave recently explored anhCGβ-targeting bladder carcinoma vaccine with a formulation that induced T- as well as B-cell mediated immune responses. It consisted of a fusion protein wherethe human monoclonal anti-mannose receptor antibody B11 was extended with hCGβ at the C-terminus (CDX-1307). In a phase II trial,CDX-1307was 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 producepredominantly 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 hCGβindicates 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 forhCGβ-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 BioPharmaInc 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 hCGβeither 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 creatingalso 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 KLHproduced significantly higher levels of immune reactive hCG antibodies than hCGβCTP-Hsp70 or hCGβCTP-KLHirrespective whether they weretiteredagainst 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 hCGβCTPformulation, 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 thissince 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 ofBAChCGβR68E orhCGβCTP 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 BAChCGβR68Eimmunogens 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 hCGβimmunogens. 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 significantlymore 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 althoughwe 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 hCGvaccines so far, the increased immunogenicity relative tohCGβCTPlooks promising.

Figures:

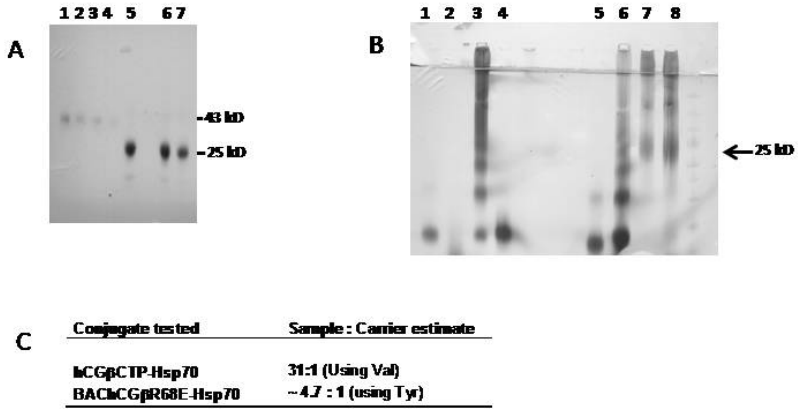

Figure 1

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 analysisusing the OT3A2 mAbshowing the coupling of hCGβCTP and BAChCGβR68E to Hsp70;Lane 1 hCGβCTP; Lane 2 Hsp70; Lane 3 hCGβCTP-Hsp70 conjugated with EDC; Lane 4 hCGβCTPmixed with Hsp70; Lane 5 hCGβCTP; Lane 6 hCGβCTP-Hsp70 conjugated with GAD; Lane 7 BAChCGβR68E-Hsp70 conjugated with EDC, andLane 8 BAChCGβR68E-Hsp70 conjugated with GAD. (**C**) Evaluation ofratio of hCGβCTP:Hsp70 and BAChCGβR68E:Hsp70 calculated from total amino acid quantification of the conjugates and Hsp70.

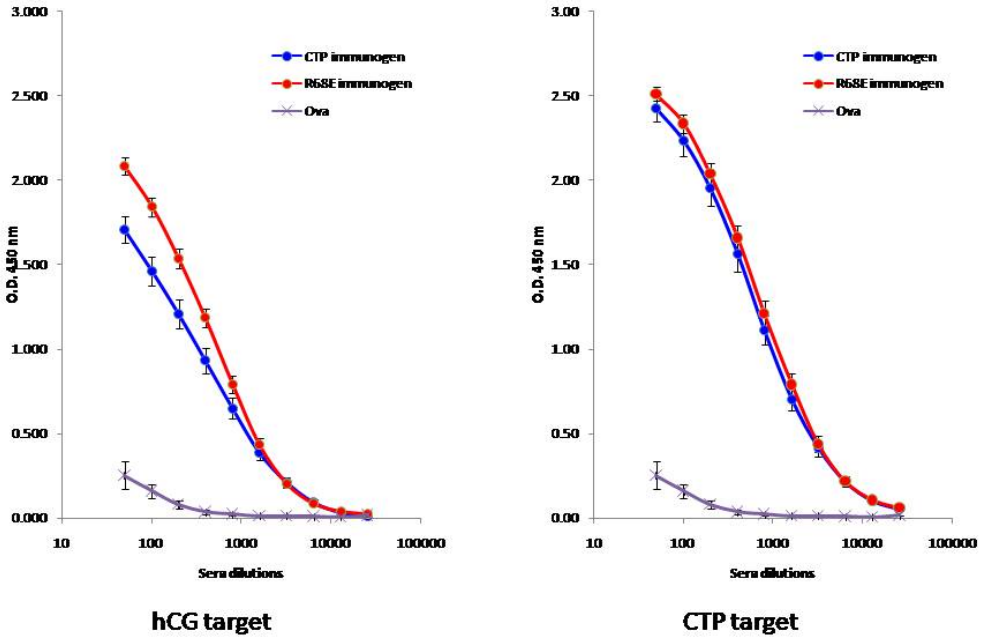

Figure 2

Figure 2 Titration of mouse immune sera. The sera from mice immunized with either hCGβCTP- or BAChCGβR68E-conjugate were endpoint titeredusing 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).

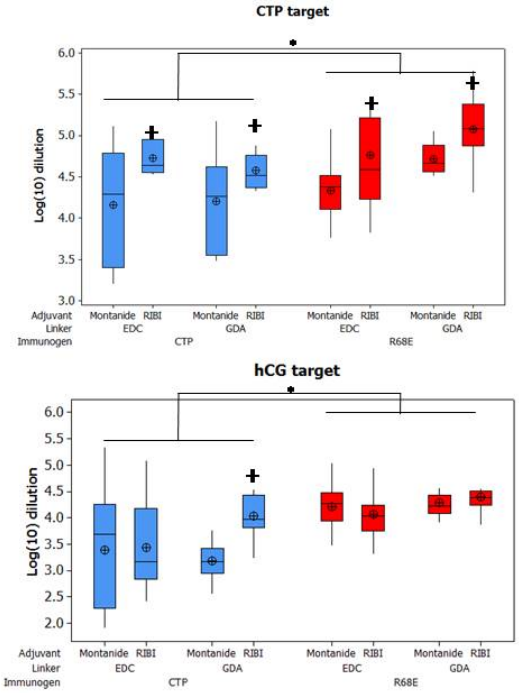

Figure 3

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 betweenBAChCGβR68E immunogen compared to thehCGβCTPimmunogen ($p<0.05$). + Indicates the significant difference in titres between the adjuvant RIBI and Montenide ISA720 ($p<0.05$).* Indicates significant differences compared to hCGβCTPimmunogen with all other conditions the same.

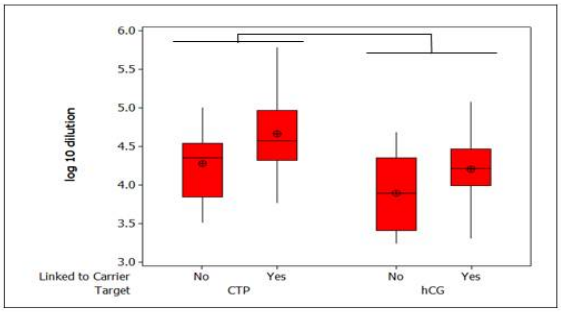

Figure 4

Figure 4 Statistical analysis of the antibody response to BAChCGβR68Eimmunogen generated as a free subunit or used when conjugated to Hsp70 or KLH all combined with the adjuvant.

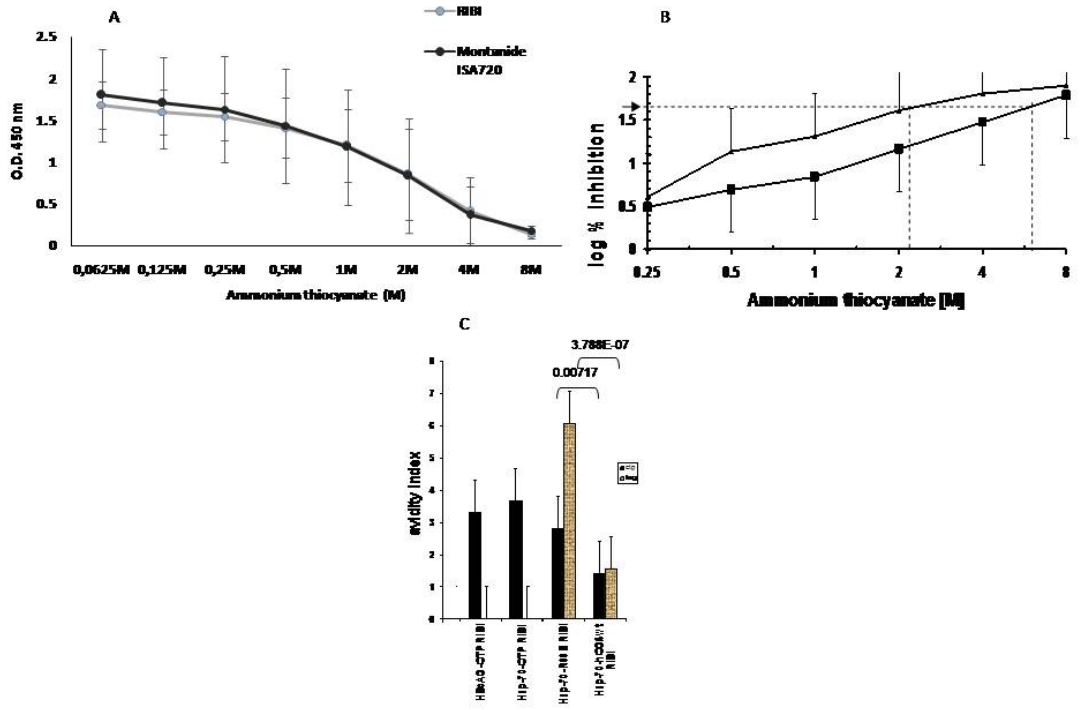

Figure 5

Figure 5 (A) Avidity of sera from the mice immunised with a BAChCGβR68Eimmunogenusing the adjuvant RIBI and Montenide ISA720 adjuvant formulationsproduce 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 titteredon 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).

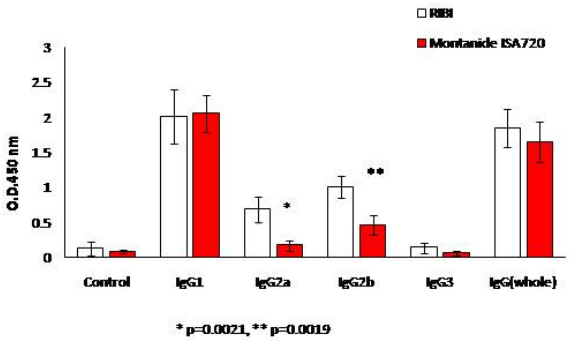

Figure 6

Figure 6 IgG subclass responses in mice immunised with the BAChCGβR68Eimmunogen using the RIBI and Montenide ISA720 adjuvant formulations and tittered on hCGβ.

Tables:

Table 1: Composition of the vaccines used to immunize the BALB/C mice

Peptide/protein	Carrier protein	Crosslinker	Adjuvant
hCGβCTP	Hsp70	GAD	Ribi
hCGβCTP	Hsp70	GAD	Montanide ISA720
hCGβCTP	Hsp70	EDC	Ribi
hCGβCTP	HSP70	EDC	Montanide ISA720
hCGβCTP	KLH	EDC	Ribi
hCGβCTP	KLH	EDC	Montanide ISA720
BAChCGβR68E			Ribi
BAChCGβR68E			Montanide ISA720
BAChCGβR68E	Hsp70	GAD	Ribi
BAChCGβR68E	Hsp70	GAD	Montanide ISA720
BAChCGβR68E	Hsp70	EDC	Ribi
BAChCGβR68E	Hsp70	EDC	Montanide ISA720
BAChCGβR68E	KLH	EDC	RIBI
BAChCGβR68E	KLH	EDC	Montanide ISA720

Table 2: Statistical analysis of the endpoint titres on ELISA on the antigens as indicated. Data represents mean (SEM) of raw data.

CTP target

Immunogen Mean (SEM)	Linker Mean (SEM)	Adjuvant Mean (SEM)
hCGβCTP 54500 (14200) n=42	EDC 62700 (20900) n=28	Montanide 35300 (10700) n=14
		RIBI 92175 (41200) n=14
	GAD 38700 (10200) n=14	Montanide 35500 (16700) n=7
		RIBI 41800 (7800) n=7
BAChCGβR68E 210800 (137300) n=42	EDC 255500 (205700) n=28	Montanide 22900 (7700) n=14
		RIBI 482000 (409700) n=14
	GAD 121300 (40900) n=14	Montanide 56800 (10900) n=7
		RIBI 185800 (75700) n=7

hCG/hCGβ target

Immunogen Mean (SEM)	Linker Mean (SEM)	Adjuvant Mean (SEM)
hCGβCTP 12600 (3300) n=80	EDC 14800 (4900) n=52	Montanide 17500 (8500) n=26
		RIBI 12100 (4900) n=26
	GAD 8500 (2000) n=28	Montanide 2000 (400) n=14
		RIBI 15000(3100) n=14
BAChCGβR68E 26500 (3600) n=84	EDC 24900 (4700) n=56	Montanide 25300 (4600) n=28
		RIBI 24400 (8300) n=28
	GAD 29900 (2000) n=28	Montanide 24900 (6300) n=14
		RIBI 34900 (9300) n=14

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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