

Middlesex University Research Repository

An open access repository of

Middlesex University research

<http://eprints.mdx.ac.uk>

Kvirkvelia, Nino, Chikadze, Nino, Makinde, Julia, McBride, Jeffrey D., Porakishvili, Nino, Hills, Frank ORCID logo ORCID: <https://orcid.org/0000-0001-8235-7545>, Martensen, Pia M., Justesen, Just, Delves, Peter J., Lund, Torben and Roitt, Ivan (2018) Investigation of factors influencing the immunogenicity of hCG as a potential cancer vaccine. *Clinical and Experimental Immunology*, 193 (1) . pp. 73-83. ISSN 0009-9104 [Article] (doi:10.1111/cei.13131)

Final accepted version (with author's formatting)

This version is available at: <https://eprints.mdx.ac.uk/24108/>

Copyright:

Middlesex University Research Repository makes the University's research available electronically.

Copyright and moral rights to this work are retained by the author and/or other copyright owners unless otherwise stated. The work is supplied on the understanding that any use for commercial gain is strictly forbidden. A copy may be downloaded for personal, non-commercial, research or study without prior permission and without charge.

Works, including theses and research projects, may not be reproduced in any format or medium, or extensive quotations taken from them, or their content changed in any way, without first obtaining permission in writing from the copyright holder(s). They may not be sold or exploited commercially in any format or medium without the prior written permission of the copyright holder(s).

Full bibliographic details must be given when referring to, or quoting from full items including the author's name, the title of the work, publication details where relevant (place, publisher, date), pagination, and for theses or dissertations the awarding institution, the degree type awarded, and the date of the award.

If you believe that any material held in the repository infringes copyright law, please contact the Repository Team at Middlesex University via the following email address:

eprints@mdx.ac.uk

The item will be removed from the repository while any claim is being investigated.

See also repository copyright: re-use policy: <http://eprints.mdx.ac.uk/policies.html#copy>

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

Nino Kvirkvelia^{1§#}, Nino Chikadze^{2§}, Julia Makinde^{1#}, Jeffrey D McBride^{1#}, Nino Porakishvili³, Frank A Hills⁴, Pia M Martensen⁵, Just Justesen⁵, Peter J Delves¹, 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 Department of 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 (Julia Makinda) 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)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 (hCG β) 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 hCG β peptide (hCG β CTP), induce antibody responses in a majority of human recipients. Here we explored whether the immunogenicity of vaccines containing an hCG β mutant (hCG β R68E, designed to eliminate cross-reactivity with luteinizing hormone) or hCG β CTP 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 BACHCG β R68E 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 hCG β CTP 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 BACHCG β R68E 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 hCG β CTP peptide vaccine. This makes this mutant vaccine a promising candidate for therapeutic studies in hCG β -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 bionutralization of hCG in women indeed prevents pregnancy [2, 3].

Highly sensitive assays have identified very low levels of hCG or hCG β 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 hCG β 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 hCG β in these cancers is still being elucidated, model systems have shown that hCG β is necessary for survival of the bladder cancer SCaBER [7] and the cervical cancer HeLa [8] cell lines. In these systems hCG β may be functioning as an anti-apoptotic growth factor [8-10]. Furthermore, high titres of hCG-specific antibody prevented the growth of an hCG β -expressing hepatoma H22 cell line xeno-grafted into mice [11]. This latter study showed also that the induced anti-hCG β antibodies significantly reduced angiogenesis in the H22 grafts. There is also evidence to implicate hCG β 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 hCG β as a cancer biomarker, identified 20 (47%) where the expression of hCG β was associated with poor prognosis and accelerated death [6]. It would seem logical therefore, that bionutralization of hCG β 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 hCG β , 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 bionutralizing hCG vaccines in humans and indeed, in the last two decades, hCG β 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 bionutralizing 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 hCG β (hCG β CTP), not present in LH β , 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 hCG β CTP covalently attached to diphtheria toxoid induced hCG-specific antibodies at levels between 0.1-2 μ g 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 O-linked glycosylation sites, which are occupied in native hCG and its free hCG β 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 hCG β target. In another approach to overcome the poor immunogenicity of hCG β CTP, Xiangbing *et al.* constructed a fusion protein consisting of heat shock protein (Hsp65) with ten tandem repeats of hCG β 109-118 and a copy of

hCG β 109-145 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 bionutralizing responses in an outbred populations like humans with their diverse HLA haplotypes.

We have previously reported an alternative hCG vaccine candidate consisting of hCG β 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 hCG β -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 hCG β CTP, 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 hCG β CTP and hCG β R68E 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 hCG β R68E is superior to CTP as a vaccine candidate.

Material and Methods

Reagents

Recombinant hCG β produced in CHO cells was purchased from Sigma-Aldrich (UK); Recombinant BACHCG β R68E was purified from baculovirus-infected HiFive insect cells (see below). The C-terminal peptide (hCG β CTP) representing the amino acid residues 108-145 of hCG β 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 hCG β -R68E

The pBAC2hCG β R68E baculovirus expression plasmid for production of recombinant hCG β R68E with a C-terminal His₆-tag [29] was transiently introduced into HiFive insect cells (InVitrogen) and a single recombinant virus expressing BACHCG β R68E 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 1.5×10^5 cells per ml at 28°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°C. Recombinant BACHCG β R68E was affinity purified in batches

of 50-200 ml insect cell supernatants after dilution with an equal volume of 20 mM Na₂HPO₄, 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 Na₂HPO₄, 0.5 M NaCl pH 7.3 followed by 20 mM Na₂HPO₄, 0.5 M NaCl, 25 mM imidazole pH 7.3, the recombinant protein was eluted with 20 mM Na₂HPO₄, 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°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 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-hCG β CTP and Hsp70-BACHCG β R68E 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 μ g aliquot of the Hsp70- or KLH-conjugate containing hCG β CTP or BACHCG β R68E 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°C overnight with 50 μ l recombinant hCG β (Sigma-Aldrich UK), hCG, or ovalbumin at 1 μ g/ml or hCG β CTP peptide at 5 μ g/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 μ l was added to

each well and incubated for 2 h at 37°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°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 washed 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 BACHCG β R68E with a molecular weight of 25 kDa is smaller than the 45 kDa CHO-produced hCG β (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 hCG β subunit fold correctly as judged by their full recognition of a panel of conformation-dependent monoclonal antibodies [30]. Once purified, BACHCG β R68E as well as synthetic hCG β CTP 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 hCG β R68E:Hsp70 and hCG β CTP: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 BACHCG β R68E and hCG β CTP to KLH due to its very large molecular weight of 7.8×10^3 kDa.

Immunogenicity of hCB β R68E versus hCG β CTP

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,

hCG β and hCG β CTP 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 hCG β** when used as target antigens. We therefore combined the results obtained with these two antigens in our statistical analysis. Given that both BACHCG β R68E and hCG β CTP 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/hCG β , the BACHCG β R68E antisera showed better binding to the antigens than the antisera elicited with hCG β CTP (for BACHCG β R68E mean titre was 1:26500; for hCG β CTP mean titre was 1:12600, $p < 0.0001$) (Table 2, Figure 3). Even when titered against the synthetic hCG β CTP peptide as the target antigen, we found that the baculovirus-derived recombinant protein elicited more potent immune response than that observed with hCG β CTP conjugates as immunogens (BACHCG β R68E mean titre 1 in 210800, hCG β CTP 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 (BACHCG β R68E versus hCG β CTP) or linker (GAD versus EDC) (Figure 3).

When considering the adjuvant system (RIBI versus Montanide ISA720) the BACHCG β R68E 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 hCG β CTP target (Figure 3). However, the CTP-vaccine did reveal differences. When titered on its biological target hCG/hCG β , 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 hCG β CTP 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 BACHCG β R68E 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 BACHCG β R68E-elicited antibodies binding to hCG β CTP with lower avidity than to hCG but with the same avidity as hCG β CTP-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 hCG β CTP immunogens and independent of the carrier when binding to hCG β CTP. In contrast, hCG β CTP-specific antibodies from hCG β CTP-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 BACHCG β R68E fixed the C-terminal part of hCG β through electrostatic interaction this masking the immunodominant LH-cross-reactive epitope on hCG β but enhances a hCG β CTP-specific epitope [29-31]. It is therefore

not surprising that the avidity of the hCG β CTP-specific antibodies were comparable to that induced by hCG β CTP-immunogens and higher than hCG β CTP antibodies induced by hCG β □□□□□□□□. In addition, the antibodies induced with at the BACHCG β R68E immunogen had a significantly greater affinity overall than antibodies induced by hCG β . 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 BACHCG β R68E vaccine formulation gave significantly greater responses against both targets than did the hCG β CTP-based vaccines (Figure 3). Using hCG as target, the hCG β CTP linked to carrier by GAD with RIBI adjuvant gave the best antibody response of the hCG β CTP immunogen group but this was significantly lower than the corresponding result with the BACHCG β R68E mutant ($p < 0.014$).

Discussion

Human CG has traditionally been associated with pregnancy, but the recent decades have revealed that hCG and hCG β are also biomarkers for trophoblastic and epithelial cancers and the presence of hCG β 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 hCG β and thereby elicit sufficient levels of antibodies to prevent pregnancy in immunized women [3]. hCG β 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 hCG β -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 hCG β 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 hCG β -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 hCG β 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 hCG β -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 hCG β . AVI BioPharma Inc has taken a vaccine consisting of hCG β CTP₃₇ 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 metastasizing colorectal carcinomas. However, the vaccine-induced hCG β CTP antibodies were not able to neutralize the tumour-derived hCG β either due to the high entropy of C-terminal segment or because the hCG β CTP 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 hCG β -producing cancers.

We show here that our hCG β R68E mutant may be a more suitable immunogen than either hCG β or hCG β CTP. 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]. BACHCG β R68E conjugated to either Hsp70 or KLH produced significantly higher levels of immune reactive hCG antibodies than hCG β CTP-Hsp70 or hCG β CTP-KLH irrespective whether they were titered against hCG, hCG β or CTP. However, the difference in the antibody levels was not as pronounced when titered against CTP. There may be several reasons for this. The molar level of CTP per Hsp70 molecule was 6.6 times higher than for BACHCG β R68E 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 O-linked carbohydrate residues present in the C-terminal part of BACHCG β R68E. One would therefore expect that not all of the antibody specificities elicited with hCG β CTP formulation, would recognize hCG/hCG β .

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 BACHCG β R68E or hCG β CTP 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 hCG β CTP, these improvements were less pronounced with BACHCG β R68E. Nonetheless, even by enhancing the immunogenicity, the hCG β CTP 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 hCG β CTP peptide-coated plates the antibodies elicited by hCG β CTP and BACHCG β R68E immunogens had the same avidity, which was

significant ($p < 0.007$ in student t-test) than hCG β CTP-specific antibodies induced by hCG β conjugated to the same carrier. However, the affinities of the antibodies produced in hCG β CTP-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 hCG β CTP 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 hCG β CTP or hCG β immunogens. This makes BACHCG β R68E a much better vaccine candidate.

In conclusion, we have compared two hCG β -specific vaccine candidates hCG β CTP and BACHCG β R68E delivered using different formulations and report here that the hCG β mutant BACHCG β R68E is a significantly more potent (or effective) vaccine than hCG β CTP 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 BACHCG β R68E 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 hCG β CTP looks promising.

Figure Legends

Figure 1. Purification of BACHCG β R68E and coupling of the immunogens to carrier proteins. (A) 1 μ l of affinity purified recombinant BACHCG β R68E was separated by 12% SDS PAGE followed by Western blotting and compared to CHO-produced hCG β : Lanes 1-3 show rhCG β at 0.25, 0.5 and 1.0 mg/ml and Lane 4-5 show two batch batches of purified BACHCG β R68E preparations. (B) Western blot analysis using the OT3A2 mAb showing the coupling of hCG β CTP and BACHCG β R68E to Hsp70; Lane 1 hCG β CTP; Lane 2 Hsp70; Lane 3 hCG β CTP-Hsp70 conjugated with EDC; Lane 4 hCG β CTP mixed with Hsp70; Lane 5 hCG β CTP; Lane 6 hCG β CTP-Hsp70 conjugated with GAD; Lane 7 BACHCG β R68E-Hsp70 conjugated with EDC, and Lane 8 BACHCG β R68E-Hsp70 conjugated with GAD. (C) Evaluation of ratio of hCG β CTP:Hsp70 and BACHCG β R68E: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 hCG β CTP- or BACHCG β R68E-conjugate were endpoint titered using direct ELISAs on plates coated with hCG (to the left) and the synthetic hCG β CTP peptide (to the right). The graphs used data that includes both linkers, both carriers and both adjuvants. They show the mean absorbance and ± 2 SD 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 hCG β CTP- or BACHCG β R68E-conjugates titered on hCG β CTP (upper diagram) or hCG/hCG β (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 BACHCG β R68E immunogen compared to the hCG β CTP 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 hCG β CTP immunogen with all other conditions the same.

Figure 4 Statistical analysis of the antibody response to BACHCG β R68E 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 BACHCG β R68E immunogen using the adjuvant RIBI and Montenide ISA720 adjuvant formulations produce antibodies with as identical ammonium thiocyanate dissociation when tittered on hCG β (* $p < 0.0021$ and ** $p < 0.0019$ Student t-test). (B) However, the dissociation of the antigen-antibody complexes in sera from mice immunised with BACHCG β R68E-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 hCG β (light). Student t-test was used to determine the statistical significance as indicated).

Figure 6 IgG subclass responses in mice immunised with the BACHCG β R68E immunogen using the RIBI and Montenide ISA720 adjuvant formulations and tittered on hCG β .

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.

Acknowledgement

We thank Professor Theo Verrips, Department of Biomolecular Imaging, Utrecht University, The Netherlands for the gift of Hsp70 and anti-Hsp70 antiserum, Professor Dr Vernon C Stephens, Ohio State University, Columbus, OH for the synthetic hCG β CTP and Dr E Bos, NV Organon, The Netherlands for the OT3A2 monoclonal antibody. We are grateful for the technical assistance of Ms Inger Bjorndahl in preparing the recombinant baculovirus supernatants and for the infections of the insect cells.

References

- [1] Fishel SB, Edwards RG, Evans CJ. Human chorionic gonadotropin secreted by preimplantation embryos cultured in vitro. *Science*. 1984;223:816-8.
- [2] Talwar GP, Singh OM, Gupta SK, Hasnain SE, Pal R, Majumbar SS, et al. The HSD-hCG vaccine prevents pregnancy in women: feasibility study of a reversible safe contraceptive vaccine. *Am J Reprod Immunol*. 1997;37:153-60.

- [3] Talwar GP, Singh O, Pal R, Chatterjee N, Sahai P, Dhall K, et al. A vaccine that prevents pregnancy in women. *Proc Natl Acad Sci USA*. 1994;91:8532-6.
- [4] Stenman UH, Alfthan H, Hotakainen K. Human chorionic gonadotropin in cancer. *Clin Biochem*. 2004;37:549-61.
- [5] Iles RK. Ectopic hCGbeta expression by epithelial cancer: malignant behaviour, metastasis and inhibition of tumor cell apoptosis. *Mol Cell Endocrinol*. 2007;260-262:264-70.
- [6] Iles RK, Delves PJ, Butler SA. Does hCG or hCGbeta play a role in cancer cell biology? *Mol Cell Endocrinol*. 2010;329:62-70.
- [7] Burczynska B, Booth MJ, Iles RK, Shah A, Shiled A, Butler SA. Stable knockdown of hCGbeta mRNA expression in bladder cancer cells results in significant growth inhibition. *Anticancer Res*. 2013;33:3611-4.
- [8] Jankowska A, Gunderson SI, Andrusiewicz M, Burczynska B, Szczerba A, Jarmolowski A, et al. Reduction of human chorionic gonadotropin beta subunit expression by modified U1 snRNA caused apoptosis in cervical cancer cells. *Mol Cancer*. 2008;7:26.
- [9] Butler SA, Ikram MS, Mathieu S, Iles RK. The increase in bladder carcinoma cell population induced by the free beta subunit of human chorionic gonadotrophin is a result of an anti-apoptosis effect and not cell proliferation. *Brit J Cancer*. 2000;82:1553-6.
- [10] Butler SA, Iles RK. Ectopic human chorionic gonadotropin beta secretion by epithelial tumors and human chorionic gonadotropin beta-induced apoptosis in Kaposi's sarcoma: is there a connection? *Clin Cancer Res*. 2003;9:4666-73.

- [11] Xiangbing H, Yankai Z, Ming L, Yong L, Yu Z, Huiyong Z, et al. The fusion protein of HSP65 with tandem repeats of beta-hCG acting as a potent tumor vaccine in suppressing hepatocarcinoma. *International Immunopharm.* 2010;10:230-8.
- [12] Wu W, Walker AM. Human chorionic gonadotropin beta (HCGbeta) down-regulates E-cadherin and promotes human prostate carcinoma cell migration and invasion. *Cancer.* 2006;106:68-78.
- [13] Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol.* 1991;113:173-85.
- [14] Sun PD, Davies DR. The cystine-knot growth-factor superfamily. *Ann Rev Biophys Biomol Structure.* 1995;24:269-91.
- [15] Laphorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, et al. Crystal structure of human chorionic gonadotropin. *Nature.* 1994;369:455-61.
- [16] Berger P, Bidart JM, Delves PS, Dirnhofer S, Hoermann R, Isaacs N, et al. Immunochemical mapping of gonadotropins. *Mol Cell Endocrinol.* 1996;125:33-43.
- [17] Berger P, Klieber R, Panmoung W, Madersbacher S, Wolf H, Wick G. Monoclonal antibodies against the free subunits of human chorionic gonadotrophin. *J Endocrinol.* 1990;125:301-9.
- [18] Berger P, Kofler R, Wick G. Monoclonal antibodies against human chorionic gonadotropin (hCG): II. Affinity and ability to neutralize the biological activity of hCG. *Am J Rerod Immunol.* 1984;5:157-60.
- [19] Bidart JM, Ozturk M, Bellet DH, Jolivet M, Gras MH, Troalen F, et al. Identification of epitopes associated with hCG and the beta hCG carboxyl terminus by

monoclonal antibodies produced against a synthetic peptide. *J Immunol.* 1985;134:457-64.

[20] Bidart JM, Troalen F, Bohuon CJ, Hennen G, Bellet DH. Immunochemical mapping of a specific domain on human choriogonadotropin using anti-protein and anti-peptide monoclonal antibodies. *J Biol Chem.* 1987;262:15483-9.

[21] Kofler R, Berger P, Wick G. Monoclonal antibodies against human chorionic gonadotropin (hCG): I. production, specificity, and intramolecular binding sites. *Am J Reprod Immunol.* 1982;2:212-6.

[22] Charrel-Dennis M, Jackson AM, Lund T, Laphorn AJ, Berger P, Roitt IM, et al. The major hormone-specific discontinuous epitopes on human chorionic gonadotrophin. *J Mol Endocrinol.* 2004;32:571-81.

[23] Jackson AM, Klonisch T, Laphorn AJ, Berger P, Isaacs NW, Delves PJ, et al. Identification and selective destruction of shared epitopes in human chorionic gonadotropin beta subunit. *J Reprod Immunol.* 1996;31:21-36.

[24] Talwar GP, Gupta JC, Shankar NV. Immunological approaches against human chorionic gonadotropin for control of fertility and therapy of advanced-stage cancers expressing hCG/subunits. *Am J Reprod Immunol.* 2011;66:26-39.

[25] Hannesdottir SG, Han X, Lund T, Singh M, Van Der Zee R, Roitt IM, et al. Changes in the reproductive system of male mice immunized with a GnRH-analogue conjugated to mycobacterial hsp70. *Reproduction.* 2004;128:365-71.

[26] Stevens VC. Antifertility vaccine. In: Perlmann P, Wigzell H, editors. *Handbook of experimental pharmacology*: Springer-Verlag; 1999. p. 443 - 61.

- [27] Moulton HM, Yoshihara PH, Mason DH, Iversen PL, Triozzi PL. Active specific immunotherapy with a beta-human chorionic gonadotropin peptide vaccine in patients with metastatic colorectal cancer: antibody response is associated with improved survival. *Clin Cancer Res.* 2002;8:2044-51.
- [28] Triozzi PL, Stevens VC, Aldrich W, Powell J, Todd CW, Newman MJ. Effects of a beta-human chorionic gonadotropin subunit immunogen administered in aqueous solution with a novel nonionic block copolymer adjuvant in patients with advanced cancer. *Clin Cancer Res.* 1997;3:2355-62.
- [29] Charrel-Dennis M, Terrazzini N, McBride JD, Kaye P, Martensen PM, Justesen J, et al. The human chorionic gonadotropin-beta arginine68 to glutamic acid substitution fixes the conformation of the C-terminal peptide. *Mol Endocrinol.* 2005;19:1803-11.
- [30] Chiesa MD, Martensen PM, Simmons C, Porakishvili N, Justesen J, Dougan G, et al. Refocusing of B-cell responses following a single amino acid substitution in an antigen. *Immunol.* 2001;103:172-8.
- [31] Porakishvili N, Chiesa MD, Chikadze N, Martensen P, Justesen J, Lund T, et al. Elimination of luteinizing hormone cross-reactive epitopes from human chorionic gonadotropin. *Vaccine.* 2002;20:2053-9.
- [32] Morse MA, Bradley DA, Keler T, Laliberte RJ, Green JA, Davis TA, et al. CDX-1307: a novel vaccine under study as treatment for muscle-invasive bladder cancer. *Expert Rev Vaccines.* 2011;10:733-42.
- [33] Ferreira MU and Katzin AM. The assessment of antibody affinity distribution by thiocyanate elution: a simple dose-response approach. *J Immun Meth.* 1995; 187:297-305.

[34] Chen w, Shen Q-X, and Bahl OP. Carbohydrate variants of the Recombinant β -subunit of the human choriogonadotrppin expressed in baculovirus expression systems. *J Biol Chem.* 1991; 266:4081-87.

[35] Morse MA, Chapman R, Powderly J, Blackwell K, Keler T, Green J, et al. Phase I study utilizing a novel antigen-presenting cell-targeted vaccine with Toll-like receptor stimulation to induce immunity to self-antigens in cancer patients. *Clin Cancer Res.* 2011;17:4844-53