

## Immune response to lytic peptides conjugated to a $\beta$ CG fragment in treated BALB/C mice

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### SUMMARY

Hecate- $\beta$ CG and Phor14- $\beta$ CG(ala) are relatively short, amphipathic alpha-helical cationic peptides with the ability to destroy selectively breast, prostate and ovarian cancer cells. Treatment with proteins and peptides frequently initiated antibody formation. Short peptides may minimize the risk of the immune system mobilization after treatment but it is necessary to investigate whether Hecate- $\beta$ CG and Phor14- $\beta$ CG(ala) induce the immune system to produce antibody and whether they affect the reproductive organs in normal wild-type mice. The results of our experiments showed that specific antibodies, tested by the enzyme-immunoassay, were not detected in the group treated with Hecate- $\beta$ CG and Phor14- $\beta$ CG(ala). The blood concentrations of both peptides begun to decrease from 60 minutes after injection and after

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240 minutes its levels were undetectable. Histopathological examination exhibited degenerative changes in the prostate glands and testes in males and in the ovaries and uteri of females treated with both peptides. In conclusion, our results indicate that both relatively small and rapidly metabolized peptides are not immunogenic and can be used for further investigation as a potential cancer treatment. *Reproductive Biology* 2008, 8, 2:135-147.

**Key words:** mice, cancer, Hecate- $\beta$ CG, Phor14- $\beta$ CG(ala), LH/hCG receptor, immunogenicity

## INTRODUCTION

Two peptides, Hecate- $\beta$ CG and Phor14- $\beta$ CG(ala), have been studied extensively as possible treatments for prostate [6], mammary gland [18] and ovarian [1] cancer. Each drug consists of a lytic peptide (Hecate or Phor14) linked to 15 amino acids from the beta chain of chorionic gonadotrophin (CG; [9]). The CG moiety of Phor14- $\beta$ CG(ala) was altered by replacing the cysteines with alanines. Their structures have been described previously [2]. Receptors for LH/hCG are expressed in androgen dependent and independent prostate cancer cells, as well as in breast and ovarian cancer cells. The lytic peptide moieties, Hecate (23 amino acids) and Phor14(ala) (14 amino acids) are amphipathic alpha-helical cationic peptides with the ability to kill targeted cells by destroying their membranes [7].

Previous data show that the cytotoxicities ( $EC_{50}$  values) of Hecate- $\beta$ CG are positively correlated ( $R^2 = 0.709$ ) with LH receptor capacity in seven different cell lines [6]. Steroid removal from the culture medium reduced sensitivity to the drug in a reversible manner [7]. Moreover, cells without LH receptors exhibited very low sensitivity to the conjugated peptide.

These data show that treatment with lytic peptides conjugated to a 15-amino acid fragment of  $\beta$ CG effectively destroy prostate [7], breast [2] and, potentially ovarian cancer cells [1] *in vitro* and *in vivo* [7, 8]. Injection of xenografted athymic nude mice with Hecate- $\beta$ CG immediately stopped growth of human cancer cells [7]. Additionally, treatment with conjugates once a week for 3 or 6 weeks reduced tumor burden and volume. The

lytic peptide-CG conjugates also have a remarkable ability to seek out and destroy human prostate and breast cancer metastatic cells [3].

Exogenous proteins and peptides are frequently approved for the clinical use; however, those compounds have the potential to cause antibody formation [14]. Hecate- $\beta$ CG was designed as a relatively short peptide (38 residues) to minimize potential immune system response after drug injection. More recently synthesized Phor14- $\beta$ CG(ala) contains only 29 amino acids [6]. Although, the reduction of amino acids diminished the ability of the peptide to kill the cancer cells, it may minimize the risk of mobilization of the immune system after treatment. Previous *in vivo* studies were conducted in nude mice that lack fully functional immune systems [5]; therefore, a question was raised as to the immunogenicity of the lytic peptide conjugates. These previous studies also revealed that all of the lytic peptide-CG conjugates adversely affected the reproductive organs, inhibiting spermatogenesis and damaging interstitial cells in the testes and inhibiting folliculogenesis and ovulation in the ovaries. No damage was observed in other organs of the treated animals, despite the fact that some of these organs have been reported to express LH/CG receptors [3]. Thus, it became important to examine the histopathologic effects of the conjugates on the reproductive organs of wild type mice. Therefore, we investigated whether Hecate- $\beta$ CG and Phor14- $\beta$ CG(ala) induce the immune system to produce antibodies and whether they affect the reproductive organs in normal wild-type mice.

## MATERIALS AND METHODS

**Animals.** Animals were treated according to The Guide for Care and Use of Laboratory Animals (NIH) and The Institutional Animal Care and Use Committee approved all procedures. Crossbred goats were used as hosts for antibody production. The goats were maintained under normal husbandry practices at the LSU Experiment Station Farm at St. Gabriel, LA. Mice 4 weeks of age, obtained from the PBRC colony of Balb/C strain mice were used in an immunogenicity experiment to check whether the lytic peptide conjugates injected intravenously initiated antibody production.

**Antibody production.** To develop a method for measuring the specific antibody content in mice during immunogenicity experiments, it was necessary to produce specific antibodies against both Hecate- $\beta$ CG and Phor14- $\beta$ CG(ala). For each conjugate, three goats (n=3) were used as hosts for antibody production. For each animal, lytic peptide conjugates (1 mg) were dissolved in 0.5 ml of water. Gradually, C-18 coupled silica particles were added to the solution, with mixing. The peptides formed their amphiphatic orientation along the C-18 polymers, providing for a water-soluble interface. Addition of C-18 particles was stopped when no more particles could be dissolved, indicating depletion of free peptide from solution. Saturated suspensions of C-18 resin and peptides were mixed with equal amounts of complete Freund's adjuvant (Sigma, USA) and emulsified. The emulsion was injected subcutaneously in at least 10 different spots within the neck area. Complete Freund's adjuvant was used for the first immunization. Booster injection was repeated twice every two weeks using incomplete Freund's adjuvant (Sigma, USA). Blood samples were collected via jugular vein from each goat 12 days after the last booster injections and allowed to clot for 24 hours at 4°C. Clotted blood was centrifuged in a Beckman GS-6R centrifuge (USA) at 3500 rpm for 30 min at 4°C. Serum was collected and filtered thorough sterile 0.22  $\mu$ m filters. The whole pool of immunoglobulins was purified from the total serum protein by a G-agarose column (Sigma, USA) according to Shimazaki et al. [15]. The protein in the eluted fractions was measured by the QuantiPro BCA Assay Kit (Sigma, USA) and the fractions with highest protein concentration (immunoglobulins) were collected and stored at -70°C.

The antibodies obtained were tested for specificity using dot blot to establish whether they were able to bind specifically to the conjugates used as an antigen. Hecate- $\beta$ CG or Phor14- $\beta$ CG(ala) in 3 different concentrations (2.4, 0.24, and 0.024 mg/ml) were spotted in 10  $\mu$ l on the Hybond-C extra membrane optimized for protein transfer (Amersham, Germany) and dried at room temperature. Dry membrane was transferred to 1% (w/v) blocking solution (Roche blocking reagent) and incubated at room temperature for 1 h. The membranes were then incubated with the suspension of antibodies diluted 1:1000 (4.5  $\mu$ g protein/ml and 3.8  $\mu$ g proteins/ml of antibodies for

Phor14- $\beta$ CG(ala) and Hecate- $\beta$ CG, respectively) in 1% (w/v) blocking solution. The membrane was then washed in PBS containing 0.1% Tween 20 (PBS-T) three times for 5 minutes. The membrane was incubated with protein G labeled with peroxidase (Zymed, USA) in a concentration of 1.5  $\mu$ g/ml for 1 h at room temperature. After the membrane was washed three times in PBS-T 20 for 5 minutes the signal was detected by the Super Signal West Pico Chemiluminescent Substrate (substrate was applied for 5 minutes and the luminescence was visualized on X-Omat film developed for 1 minute).

### **Enzyme-immunoassay**

**Measurement of lytic peptide conjugates concentration.** Purified immunoglobulins anti Hecate- $\beta$ CG or Phor14- $\beta$ CG(ala) were used to develop an enzyme-immunoassay for measuring the serum concentrations of both lytic peptide conjugates. For the standard curve, 96 well plates were coated with 200  $\mu$ l of Hecate- $\beta$ CG or Phor14- $\beta$ CG(ala) in coating buffer (50 mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub>; 0.1% sodium azide). Concentrations of conjugates in the standard curve ranged from 5-39  $\mu$ g/ml in serial dilutions. After 1 h of incubation, the wells were washed with the washing buffer (PBS-T) and incubated for 30 min at 37°C with blocking solution (Roche, Switzerland). Next, antibodies diluted in blocking solution (1:1000) were applied and incubated for 1 h in 37°C. After washing three times with washing buffer, a 200  $\mu$ l of protein G labeled with peroxidase were applied in 5 ng/ml concentration for 1 h in 37°C. For the detection of peroxidase, 1 mg/ml of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid; Boeringer Mannheim, Germany) was added for 1 h incubation in the recommended 1mg/ml concentration. The color of the samples and standards was measured at 405 nm in a plate reader. This enzyme-immunoassay was also used for the cross reactivity measurements with Hecate-, Hecate, Phor14- $\beta$ CG(ala), Phor14,  $\beta$ CG, LH, FSH, and LHRH as antigens.

**Measurement of antibody concentration.** 96 well plates were coated with 1 ng/ml of Hecate- $\beta$ CG or Phor14- $\beta$ CG(ala), dissolved in coating

buffer, covered tightly with plastic film and incubated for 1 h at 37°C. The plates were then washed with PBS-T buffer and incubated for 30 min at 37°C with the blocking solution (Roche, Switzerland). Antibodies purified on protein G-Agarose columns were used to construct a standard curve to measure concentrations of specific antibodies in mouse plasma after injection of lytic peptides. Dialyzed goat's antisera against Hecate-βCG or Phor14-βCG(ala) were added to the 96 well plates in a range of 0.003-3000 ng/ml of protein (dilutions of specific antiserum from 1:1 to 1:1,000,000 were used) and incubated for 1 h at 37°C. Then, the plates were washed with the washing buffer and 1.5 μg/ml of Protein G (Roche, Switzerland) labeled with peroxidase was added and incubated for 1 h at 37°C. Peroxidase was determined colorimetrically at a wave length of 405 nm after adding 1 mg/ml of ABTS (Boeringer Mannheim, Germany) and incubated for 1 h.

**Experiment I.** The primary goal of this experiment was to determine whether the immune systems of normal Balb/C mice responded to therapeutic doses of lytic peptide conjugates. Mice at 6 weeks of age (25-30 g body weight) were injected with Hecate-βCG or Phor14-βCG(ala) into the lateral tail vein at a dose of 12 mg/kg of body weight. The control mice received an equal volume of saline (0.1 ml). Animals treated with each peptide were divided into two groups, each consisting of 6 females

*Table 1.* Cross binding of antibody obtained from selected goats after Phor14-βCG(ala) or Hecate βCG administration.

Peptides and hormones	Anti Phor14-βCG(ala) (Goat #468)	Anti Hecate-βCG (Goat #552)
Hecate-βCG	25%	100%
Hecate	27%	12.3%
Phor14-βCG(ala)	100%	10.4%
Phor14	24%	4.2%
βCG	0%	0%
LH	1.6%	1.57%
FSH	0%	0%
LHRH	0%	0.6%

and 6 males. They were injected with the compound once a week for 3 weeks. Then, after a 4-week break, mice were again injected with the same dose once a week for 3 weeks. Body weights were determined weekly. Seven days after the last injection, the mice were euthanized and blood was collected for measuring specific antibody concentrations, and complete necropsies were performed. Total serum immunoglobulins were purified by protein G-Agarose and protein concentrations in the purified serum were determined. Next, mice serum were added to the 96 well plates in a range of 0.3-3000 ng/ml of protein and specific antibody for Hecate- $\beta$ CG or Phor14- $\beta$ CG(ala) were measured by enzyme-immunoassay.

A secondary goal was to histopatological examination of collected organs including: liver, spleen, heart, kidney, adrenal gland, pancreas, lung, prostate, testes, ovaries and uteri. Collected tissues were immediately fixed in 10% buffered formalin. Following fixation for several weeks, the tissues were trimmed, embedded in paraffin, sectioned (6 microns in thickness) and stained with hematoxilin and eosin. The sections were examined using a Zeiss Universal microscope (Germany) with 3, 6, 16 and 40 $\times$  objectives.

**Experiment II.** This experiment was conducted to determine the rate at which lytic peptide conjugates are metabolized and how long the drugs are detectable by enzyme-immunoassay in the blood. Normal Balb/C mice at 6 weeks of age were divided into 18 experimental groups of 10 mice each. Hecate- $\beta$ CG or Phor14- $\beta$ CG(ala) were injected into the lateral tail vein (12 mg/kg of body weight). The first group of mice was sacrificed immediately after drug injection and remaining groups of animals were subsequently sacrificed after 15, 30, 45 minutes and 1, 1.5, 2.5, 4, and 6 hours after injection. Immediately after euthanasia blood was collected by cardiac puncture for measuring the concentration of Hecate- $\beta$ CG or Phor14- $\beta$ CG(ala).

### **Statistical analysis**

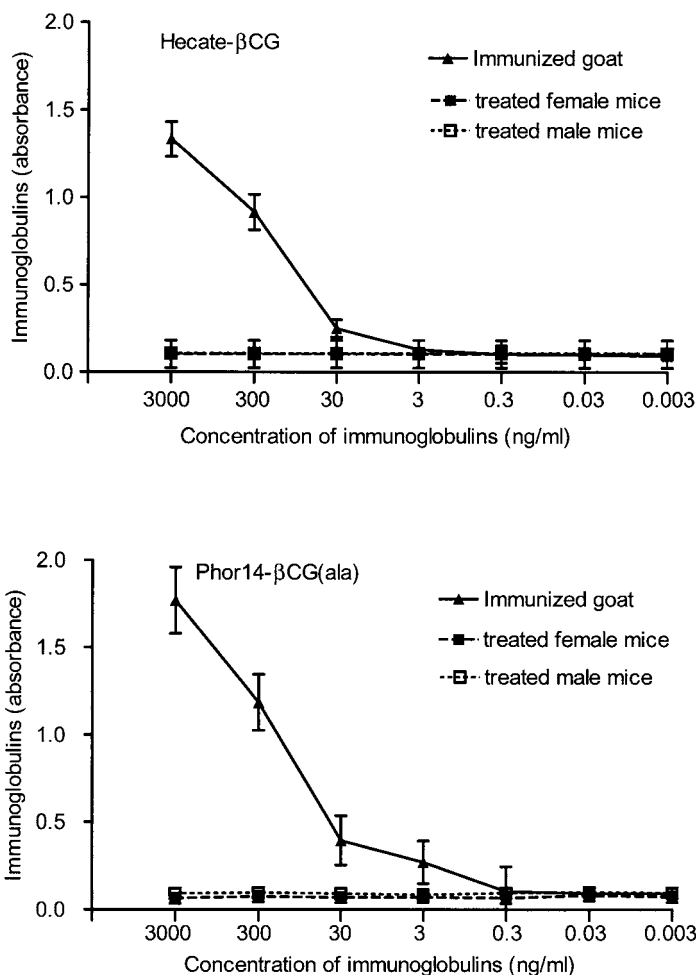
All data are presented as means  $\pm$  SEM. Statistical analyses were performed using Statistica (version 6, StatSoft Inc, Tulsa, OK USA). Significant

differences were established by one-way Anova with least significant differences (LSD) *post hoc* test and assumed as statistically significant for  $p \leq 0.05$ .

## **RESULTS**

In experiment I, purified antiserum from Hecate- $\beta$ CG immunized goats formed a linear standard curve ranging from 3 to 3000 ng/ml (fig. 1A). Values below 3 ng/ml were not detectable by the enzyme-immunoassay. Purified antiserum of Phor14- $\beta$ CG(ala) immunized goats also formed a linear standard curve ranging from 0.3 to 3000 ng/ml (fig. 1B). Specific antibodies were not detected by the enzyme-immunoassay in either treatment group (fig. 1A and 1B). Absorbance values for undiluted and diluted serum samples were below the detection level of both peptide assays.

Histopathological examination showed degenerative changes in the prostate glands and testes of all males and in the ovaries and uteri of all females treated with Phor14- $\beta$ CG(ala). Changes in the testes consisted of diminished numbers of interstitial cells; the remaining interstitial cells demonstrated intense cytoplasmic eosinophilia and pyknotic, deeply basophilic nuclei. Both changes are consistent with severe cellular degeneration and/or necrosis. In addition, 60-75% of the seminiferous tubules demonstrated severe vacuolar degeneration of the epithelium and a marked depletion of spermatids and spermatocytes. The prostate glands in these mice consisted of markedly thinned and vacuolated granular epithelium with little to no secretory product in the lumen of the glands. These changes are consistent with severe degeneration and necrosis and indicate glandular atrophy. The seminal vesicles, on the other hand, were not significantly different from the controls. In the Phor14- $\beta$ CG(ala) treated females, the ovaries were significantly reduced in size; few primary, secondary and tertiary follicles were present. Ovarian follicles and corpora lutea contained numerous necrotic cells with considerable cellular debris and many small pyknotic cells. The uteri of treated females were diminished in diameter with



*Figure 1.* Specific immunoglobulins for Hecate-βCG and for Phor14-βCG(ala) in the serum of immunized goats (solid lines) and treated mice (dashed lines) with either lytic peptide. The serum in the assay was used in a serial dilutions from 3000 ng/ml to 0.003 ng/ml of total immunoglobulins.

marked thinning of both the mucosa and muscular layers. Uterine glands were severely diminished in number and contained no secretory products. Changes in the Hecate-βCG treated males and females were generally the same as in those treated with Phor14-βCG(ala). However, there was a greater variation in the severity of the lesions in the males.

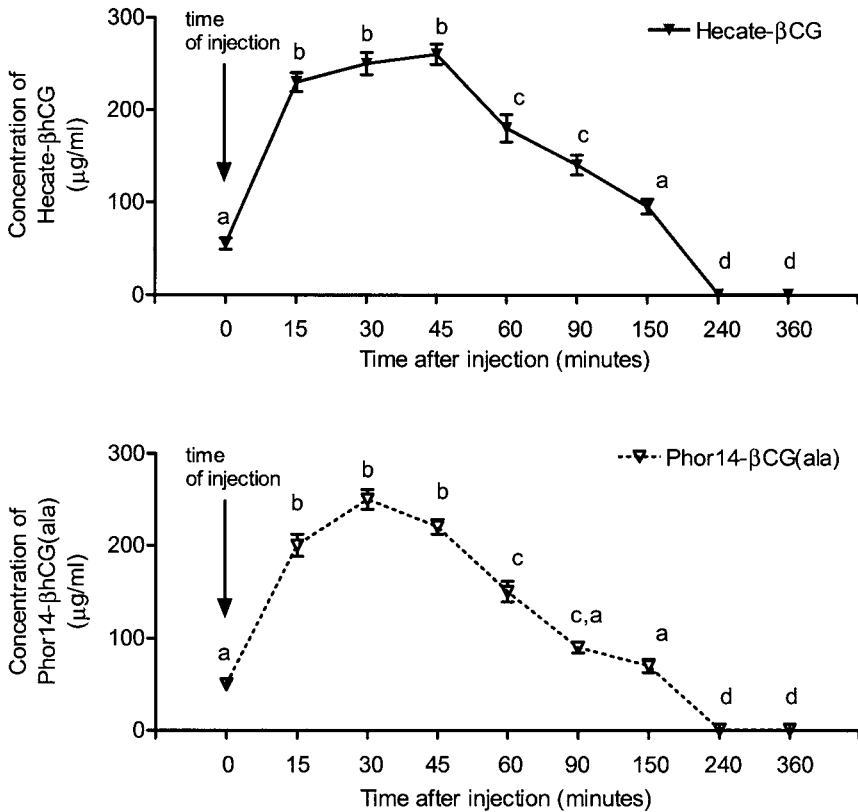


Figure 2. Serum concentration of Hecate βCG and Phor14-βCG(ala) immediately and in various time after injection of either lytic peptide into mice tail vein. Different letters in the time points mean statistical differences ( $p < 0.05$ ).

In experiment II, the concentration of Hecate-βCG in the blood (fig. 2A) increased within 15 minutes from the injection time to the highest level ( $p < 0.01$ ) and was elevated until the 45th minute after treatment. The concentration was decreased at 60 and 90 minutes compared to the 15th, 30th and 45th minutes after injection time ( $p < 0.01$ ). At 150 minutes the level of Hecate-βCG decreased to that measured immediately after injection ( $p > 0.05$ ). At the 240th and 360th minutes after injection the Hecate-βCG concentration decreased to an undetectable level ( $p < 0.01$ ).

In experiment II, the blood Phor14-βCG(ala) concentration (fig. 2B) increased within 15 minutes from injection time to the highest level

( $p < 0.01$ ) and was elevated until 45 minutes. In the 60th and 90th minutes after injection, the concentration was decreased compared to the 15th, 30th and 45th minutes after injection time ( $p < 0.01$ ). At 90 and 150 minutes from injection, the concentration of Phor14- $\beta$ CG(ala) was at the level measured immediately after injection ( $p > 0.05$ ). At 240 and 360 minutes after injection, the Phor14- $\beta$ CG(ala) concentration decreased to an undetectable level ( $p < 0.01$ ).

## DISCUSSION

Many drugs are highly immunogenic, what decrease their efficacy as therapeutic agents [14]. On the other hand, neutralization of antibodies or suppression of the immune system to reduce the production of antibodies are also likely to cause loss of therapeutic efficiency [12]. Our data indicate that neither Hecate- $\beta$ CG nor Phor14- $\beta$ CG(ala) caused antibody production during the experimental period (11 weeks from the first and 1 week from the last injection of the peptides, figures 1 and 2). Antibodies were not detected by the enzyme-immunoassay after a treatment schedule that was identical to that used in previous experiments that resulted in destruction of prostate and breast cancer tumors [2, 3].

The immunogenicity of therapeutic proteins/peptides can be influenced by many factors, including the genetic background of the patient, the type of disease, the type and size of protein, the presence of conjugates or fragments, the route of administration, dose frequency, and duration of treatment. Therefore, the peptides Phor14- $\beta$ CG(ala) and Hecate- $\beta$ CG used in our study were constructed with the smallest possible number of amino acids. Obtained results indicate that these constructs with a limited number of amino acids in the chain did not mobilize the immune system to produce specific antibodies. These results are consistent with the results of previous experiments showing that these conjugates were highly effective for targeted cells [2, 3].

Generally, proteins and peptides administered intravenously, intraperitoneally or orally are less immunogenic than those administered

by the subcutaneous or intradermal routes [10, 11, 13]. Data from four patients in which the immunogenicity of IFN- $\beta$  was studied show higher antibody formation after subcutaneous and lower after intramuscular administration [4].

In contrast to proteins that need to be present in the circulating blood for a long period of time to achieve their therapeutic effects, the amphipathic peptides used in our studies are completely metabolized within 240 minutes. This short time of exposure of the peptides to the immune cells may decrease the probability for antibody formation.

Since most previous experiments using Hectate- $\beta$ CG and Phor14- $\beta$ CG(ala) as cancer treatments were conducted on nude athymic mice that have impaired immune systems, it was important to examine the immunogenicity of these conjugates in wild type animals, which have been shown to be an appropriate model for testing relative immunogenicity of plasminogen analogs [16]. Determination of the incidence and amount of IgG antibody produced in response to injections of the molecule into the animals is the best method for evaluating immunogenesis [17]. Our experiments indicate that these relatively small, rapidly metabolized peptides are not immunogenic and can be used for further investigation as a potential cancer treatment. As expected, the ovaries, uteri and testes, which express large number of LH/CG receptors, were damaged by both drugs. A number of other chemotherapeutic cancer treatments cause damage to the testes and ovaries resulting in infertility.

In conclusion, presented results indicate that both Hectate- $\beta$ CG and Phor14- $\beta$ CG(ala) as a relatively small peptides are rapidly metabolized and not immunogenic. Therefore, they can be used for further investigation as a potential cancer treatment.

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