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Computer-aided design and physiological testing of a luteinising hormone-releasing hormone analogue for 'adjuvant-free' immunocastration

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An analogue of LHRH containing an extension of Gly-Cys at the carboxyl-terminus has been designed to permit reproducible coupling to a suitably modified carrier via a thioether bond. Potential energy calculations indicated that this analogue adopted a conformation in solution virtually identical to the type II′ turn around Gly-6–Leu-7 predicted for native LHRH. Intradermal administration of a conjugate of this analogue with purified protein derivative of tuberculin to male rats previously primed with BCG vaccine rapidly led to complete testicular regression. This adjuvant-free immunisation protocol may represent an alternative to castration for the veterinary control of reproductive function.

Luteinising hormone-releasing hormone; Structure prediction; Molecular design; Immunological castration

1. INTRODUCTION

Luteinising hormone-releasing hormone (LHRH, Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is secreted by the hypothalamus and is responsible for controlling the release of luteinising hormone and follicle stimulating hormone from the pituitary [1,2]. Active immunisation using a conjugate of the peptide with a suitable carrier in Freund's complete adjuvant (FCA) leads to the production of high titres of anti-LHRH antibodies capable of marked physiological effects in a number of species [3–5]. These effects include a reduction in sex steroid levels, arrest of gametogenesis and atrophy of gonadal tissues (review [6]).

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Autoimmunisation to LHRH may represent an alternative route to castration for the veterinary control of reproductive function [7] and for the clinical control of prostate carcinoma [8]. The practical realisation of such an approach has previously been frustrated by the need for deleterious adjuvants such as FCA, which are proscribed for clinical or veterinary use. In this paper an alternative, 'adjuvant-free', immunisation protocol based on the conjugation of a novel analogue of LHRH with purified protein derivative (PPD) of tuberculin [9] is described.

2. MATERIALS AND METHODS

2.1. *Computational methods*

The conformational preferences in solution of LHRH and an analogue, LHRH-Gly-Cys-OH, were studied using the LUCIFER program for potential energy administration [10]. The program was run on the CDC Cyber 205 computer at UMRCC. Solvent effects were modelled using the

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representation previously used for the study of neurotensin [11].

2.2. *Synthesis of LHRH analogue*

LHRH-Gly-Cys-OH was synthesised by Cambridge Research Biochemicals and supplied at 85% purity.

2.3. *Preparation of conjugates*

LHRH-Gly-Cys-OH-PPD: 10 mg of bovine PPD (Batch no.291, Central Veterinary Laboratory, Weybridge) was dissolved in 0.5 ml of 0.05 M $NaKPO_4$, 0.14 M NaCl, pH 7.0 (buffer A), and treated with 1 mg *N*-γ-maleimidobutyryloxysuccinimide (Calbiochem) previously dissolved in 5 μl freshly distilled, dry dimethyl formamide. The mixture was stirred for 1 h at 23°C and applied to a column of Sephadex G25 (0.9 × 25 cm) equilibrated with buffer A. The modified PPD eluting in the excluded volume was transferred to a stoppered vessel and to it was added dropwise, with stirring, 10 mg of LHRH-Gly-Cys-OH dissolved in 1 ml of buffer A previously degassed and purged with nitrogen. The mixture was stirred at 23°C under nitrogen for 2 h and the amount of LHRH which bound to the PPD determined by estimating the free thiol content of the mixture at regular intervals using 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) according to Deakin et al. [12]. A coupling efficiency of 65% was noted. The conjugate was extensively dialysed against several changes of distilled water at 4°C, lyophilised, and stored at −20°C before use. Tetanus toxoid (TT) (Wellcome) and bovine serum albumin (BSA) (Sigma) were conjugated to LHRH-Gly-Cys-OH exactly as described for PPD above.

2.4. *Animals*

Male (AO × DA) F_1 rats, 4 months old, were obtained from the animal unit, University of Manchester Medical School.

2.5. *Immunisation*

Groups of 5 rats were immunised with an amount of conjugate equivalent to 50 μg LHRH-Gly-Cys-OH according to the following schedule. Groups 1 and 2 received LHRH-Gly-Cys-OH-PPD intradermally in saline. Groups 3 and 4 received LHRH-Gly-Cys-OH-PPD as an alum precipitate subcutaneously. Groups 5 and 6 received LHRH-Gly-Cys-OH-TT subcutaneously in alum or FCA respectively. Group 7 received no immunisation. Groups 1 and 3 received BCG vaccine (Glaxo) equivalent to one half the human dose 1 month prior to the first immunisation with conjugate. Immunisation consisted of a primary injection followed by two booster injections at 1 monthly intervals. Animals were bled by cannulation of the tail artery at fortnightly intervals for estimation of antibody titres, and then were killed 3 months after the primary immunisation, the testes removed, weighed and subjected to histological examination. Estimation of anti-LHRH antibody titre was achieved by ELISA using plastic microtitre plates coated with LHRH-Gly-Cys-OH-BSA. Assays were developed with 1:1000 dilution of goat anti-rat IgG alkaline phosphatase conjugate.

2.6. *Histology*

Testes were fixed in neutral buffered formalin, embedded in paraffin wax and 5 μm sections cut. Sections were stained with haematoxylin and eosin.

3. RESULTS

3.1. *Conformational studies*

From an extensive conformational analysis of mammalian LHRH, a range of conformers were calculated within 7.5 kcal/mol of the predicted global minimum [13]. The lowest energy solution conformer identified has a type II′ turn around Gly-6 and Leu-7 (fig.1a). This compact structure resembles that proposed by Struthers et al. [14] from a molecular dynamics study, and is compatible with the activity of analogues *N*-methylated at Leu-7, constrained at the Gly-Leu linkage by a lactam ring, or containing D-enantiomer substitutions at position 6 [15].

While any of the predicted low energy conformers could represent the receptor-bound, bioactive form, the Boltzmann distribution indicates that the lowest energy form would predominate in solution, and therefore it is against the three-dimensional surface features of this conformer that the bulk of antibodies will be raised.

A design method previously employed is to energy minimise a range of analogues and to correlate relative biological potencies with the relative

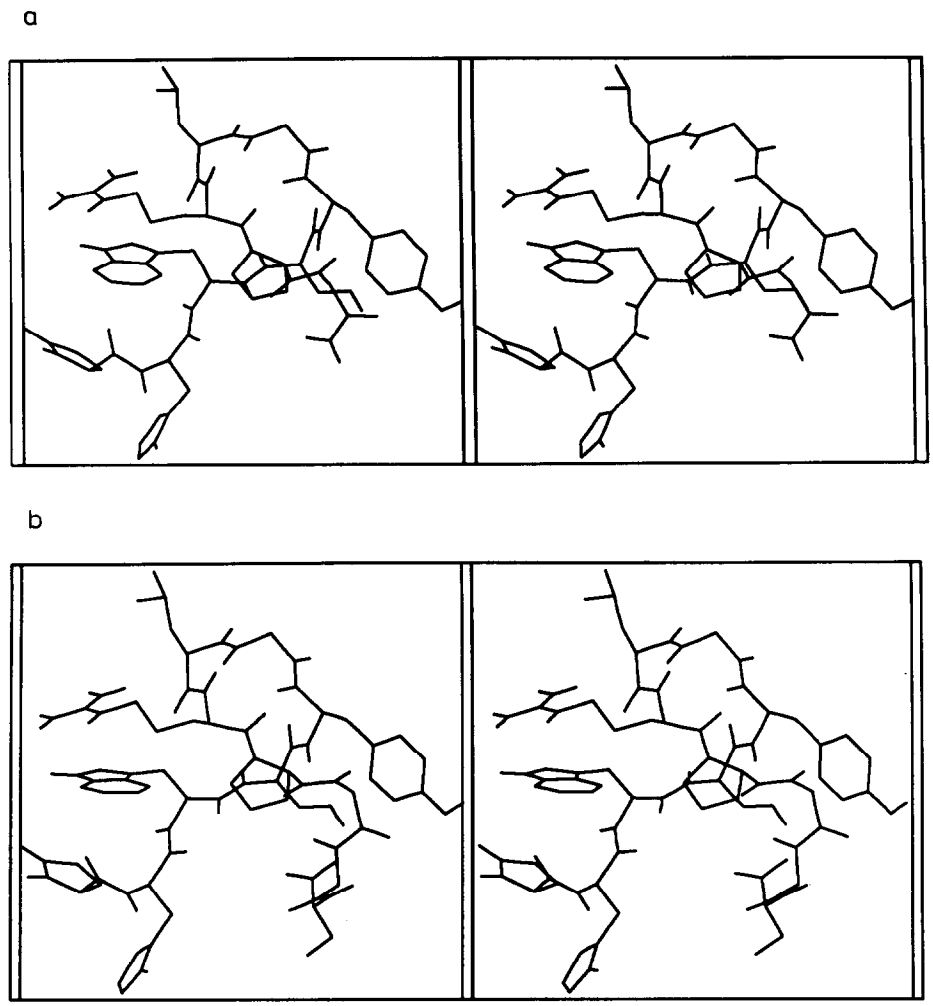

Fig.1. Stereoscopic diagrams of the predicted lowest energy solution conformers of (a) mammalian LHRH, and (b) the LHRH-Gly-Cys-OH analogue.

energetic preferences of the analogues for particular conformer classes [16]. An extension of this approach is that the predicted global minimum structure of the native peptide can be used as a 'template' to test energetic preferences on inclusion of active residue substitutions [17]. In the present case a C-terminal extension of Gly-Cys was added to the template, and after energy minimisation little deviation in overall arrangement of the molecule was noted (fig.1b). The ability of antibodies raised against the LHRH-Gly-Cys-OH-PPD to cross-react with endogenous LHRH supports this prediction in gross terms, with the reservation that the effects of the carrier were not included in the simulation.

3.2. *Antibody titres to LHRH*

Each of the groups of rats immunised with LHRH-Gly-Cys-OH conjugates gave an antibody response to the hormone detectable by ELISA. Fig.2 shows antibody titres at week 10 for each group. Immunisation with the conjugate of LHRH-Gly-Cys-OH-TT led to significant antibody levels, and these were higher in the group receiving conjugate in FCA. Although all four groups of rats receiving LHRH-Gly-Cys-OH-PPD

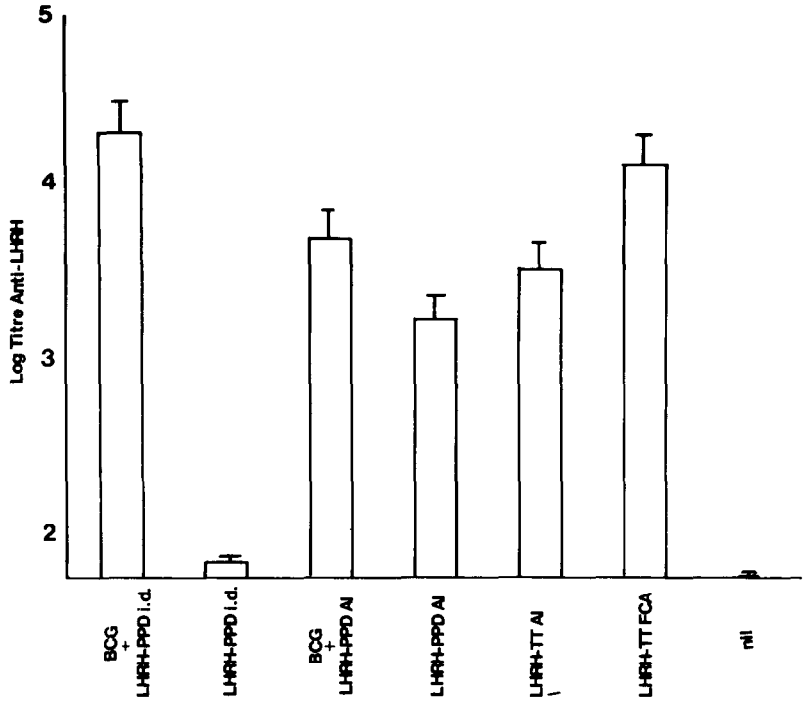

Fig.2. Day 70 anti-LHRH-Gly-Cys-OH antibody titres in rats immunised with various LHRH-Gly-Cys-OH conjugates.

gave antibody responses, responses in those groups (1 and 3) previously primed with BCG titres were considerably higher than in those which were not (2 and 4). LHRH-Gly-Cys-OH-PPD given intradermally was a very poor immunogen in normal rats, but in the BCG-primed animals led to the highest titre of antibody seen in any of the groups. LHRH-Gly-Cys-OH-PPD absorbed onto alum and given subcutaneously led to significant antibody production in normal rats, but in the BCG-primed rats the response was inferior to that obtained with the soluble antigen given intradermally.

3.3. *Physiological effects of immunisation to LHRH*

Mean testis weights for individual rats in each group are shown in fig.3. Marked testicular regression was apparent in all members of the group receiving soluble LHRH-Gly-Cys-OH-PPD after BCG priming and also in the group treated with LHRH-Gly-Cys-OH-TT in FCA although, here, one animal showed no effects. Less marked effects were seen in group 3 (2 of 5) and group 5 (1 of 5). Histological examination of testes revealed that in those individuals exhibiting severe regression there was evidence of marked atrophy of seminiferous tubules and absence of spermatogenesis.

4. DISCUSSION

The autoimmunisation protocol presented here has two advantages over previous procedures. Firstly it does not rely on proscribed adjuvants, and therefore is potentially suitable for clinical and veterinary applications. Secondly, the preliminary results would indicate that this schedule is more effective than other approaches, since rapid and complete testicular regression was noted in all five animals receiving the new treatment.

The efficacy of the new procedure may be partly attributable to the method of conjugation employed. Hitherto published procedures for the conjugation of LHRH to a carrier have generally involved the condensation of the native peptide with a water-soluble carbodiimide (WSC). The

Fig.3. Effect of immunisation with various LHRH-Gly-Cys-OH conjugates on testicular weight in rats.

absence of free carboxyl or amino moieties in LHRH means that coupling is probably effected through the hydroxyl group of Ser-4, or via a carboxymethylated derivative of His-2. As a result the immunogen is poorly defined, and there is a danger that the peptide is attached through a region important for the immunological recognition of the endogenous hormone.

The novel analogue employed here contains an extension of Gly-Cys at the C-terminus to permit reproducible coupling to the carrier via the cysteine side chain. The conformational studies have been valuable as a design tool by showing that the analogue maintains the type II′ turn predicted for the native peptide, and although it is possible that conjugation will influence the solution structure of the analogue, the extension is both flexible and of sufficient length to hold the peptide away from the surface of the carrier.

PPD of tuberculin is an excellent carrier for peptide antigens when used in recipients previously primed with BCG [9]. The mechanism of action is not known, but it is most likely that it is the induction of a local inflammatory reaction to the carrier which leads to a particularly vigorous response to the peptide. Nevertheless, the use of the extended analogue would seem to be crucial for the success of these experiments, since a conjugate of LHRH and PPD prepared using the native peptide and WSC was unable to induce a physiological response in immunised animals (not shown). D-Lys-6-LHRH conjugated to PPD with glutaraldehyde has likewise been found to be ineffective. It should also be noted that the intradermal route of immunisation appears to be important: the same antigen injected as an alum suspension was less effective at immunocastration.

The advantages of the immunisation schedule described above make this procedure potentially suitable for clinical or veterinary use. Although a totally synthetic LHRH vaccine based on muramyl dipeptide (MDP) has recently been shown to effect immunological castration in male mice [18,19], the apparent pyrogenic effects of MDP may prove to be a drawback with this approach. Further studies on the dose requirements for the LHRH-Gly-Cys-OH-PPD procedure are currently in progress, and will be presented elsewhere.

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