Changes in the reproductive system of male mice immunized with a GnRH-analogue conjugated to mycobacterial hsp70

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Abstract

Immunosterilization is an attractive alternative to surgical castration. Gonadotropin-releasing hormone (GnRH) controls the production of the gonadotropins thereby having an orchestrating effect on the reproductive hormone cascade and spermatogenesis. Induction of neutralizing antibody can abrogate the effect of the hormone. Current GnRH-based vaccines often require strong adjuvants and/or multiple injections of the vaccines to overcome variability in the response. Heat shock proteins (hsp) have been used as carrier molecules because of their powerful intrinsic ability to enhance an immune response to associated antigens. A GnRH-analogue, GnRH-d6-Lys, was conjugated to recombinant Mycobacterium tuberculosis hsp70. Male BALB/c mice were immunized i.p. with GnRH-hsp70 in the mild adjuvant Ribi or in incomplete Freund's adjuvant (IFA). The initial immunizations were done on pre-pubertal 3-week-old mice, with boosts at 5 and 8 weeks of age. The mice were killed at 10 weeks of age and GnRH-specific antibodies and serum testosterone levels measured. All the immunized mice mounted GnRH-specific antibody responses, with no difference in the mice immunized with GnRH-hsp70/Ribi or with GnRH-hsp70/IFA. There was substantial atrophy of the urogenital complex and significantly (P < 0.0005) reduced levels of testosterone-dependent testicular relaxin-like factor mRNA expression. Mice immunized with GnRH-hsp70/Ribi showed substantially reduced (P < 0.001) serum testosterone levels. These results indicate that hsp70 may serve as a particularly advantageous carrier for GnRH-based vaccines.


Introduction

Reproduction and sexual behaviour in mammals is governed by various hormonal, neurological and social factors. Farmed animals, including animals reared for food production, along with companion animals are routinely castrated for a number of reasons. For example, castration of boars permits increased growth and avoids the unpleasant odour of fat (‘boar taint’) associated with male hormone breakdown products. Surgery to sterilize larger animals such as horses has a risk of infections and morbidity (Mee et al. 1998). Various alternatives to surgical procedures have been proposed, among them the use of a specific immunosterilization vaccine targeted at one or more factors of the reproductive system. Hormones of the reproductive system are prime candidates for such vaccines as they govern the production of sex steroids and gametes in the gonads. To be effective, such a sterilization vaccine needs to be reliable, convenient (i.e. less laborious than surgical procedures), cost effective and safe.

The reproductive hormone cascade is governed by a hypothalamic peptide, gonadotropin-releasing hormone (GnRH). It is secreted in a pulsatile manner into the pituitary portal vessels and acts upon gonadotrophs in the pituitary to stimulate the production of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Previous immunosterilization vaccines utilizing GnRH have proved that provocation of an immune response to GnRH can lower gonadotropin and testosterone levels and inhibit spermatogenesis (Ferro 2002). In addition to utility as immunosterilizing agents in animals, vaccines based on GnRH have potential in the treatment of human cancers such as carcinoma of the prostate (Ferro 2002). One limiting factor in the use of GnRH vaccines is that there is usually considerable variation in the incidence and level of response to immunization. Furthermore, the traditional adjuvants that have been used, e.g. complete Freund's adjuvant (CFA), are toxic and have various adverse side effects (Leenaars et al. 1998). It is therefore important
to develop a vaccine that is efficient in all individuals tested and can be given in an acceptable adjuvant.

GnRH in its native form, being a short peptide and a self-hormone, is very poorly immunogenic. Conjugation of the peptide to large carrier molecules such as tetanus toxoid (TT), diphtheria toxoid (DT) and branched polylysine overcomes this problem and induces antibodies in immunized animals (Ladd et al. 1990, Sad et al. 1991, Beekman et al. 1999, Ferro et al. 2002). Carrier conjugation not only supplies the peptide with T cell epitopes necessary for an efficient antibody response, but also has the advantage that less of the peptide is needed to raise an immune response. A usual prerequisite for the peptide/carrier collaboration is that the peptide is covalently linked to the carrier either chemically or by co-expressing the peptide along with the carrier molecule using recombinant DNA technology. Irrespective of the method chosen it is paramount to conserve any natural conformation of the peptide epitope, so that antibodies recognise the native form of the peptide.

A disadvantage of some carrier molecules is their relatively low immunogenicity and the need for potent adjuvants such as CFA to stimulate the immune response non-specifically. Various systems are being developed to circumvent the need for strong and often toxic adjuvants. Approaches include Pam2Cys-based GnRH lipopeptides (Zeng et al. 2002) and the use of CpG oligonucleotides with a retro-inverso peptide/dimetric of GnRH (Fromme et al. 2003). Certain carrier proteins such as the mycobacterial heat shock protein (hsp) 70 also have adjuvant-like properties and may be used efficiently as carriers in an adjuvant-free system. Epitope analysis has shown that hsp70 proteins have numerous B and T cell epitopes (Peake et al. 1993, Requena et al. 1993, Quijada et al. 1996) and the hsp70 from M. tuberculosis can evoke a strong T cell dependent immune response without the need for external adjuvant when used as a carrier molecule coupled to a peptide antigen (Lussow et al. 1991, Lehner et al. 2000, van Eden et al. 2003). ATP-treated hsp70 can be either loaded with (Ciupitu et al. 1998), or covalently linked to (Suzue and Young 1996), peptides to elicit specific anti-peptide immune responses. Its inherent adjuvanticity is mediated, at least to some extent, by induction of β-chemokines (RANTES, MIP-1α and MIP-1β) produced by CD8+ T-cells (Lehner et al. 2000). The β-chemokines in turn attract dendritic cells, macrophages and both CD4+ and CD8+ T-cells (Hedrick and Zlotnik 1996). Intriguingly, hsp70 can directly activate dendritic cells by ligating the CD40 antigen (Wang et al. 2001).

We wished to investigate whether the use of hsp70 could provide a means of developing a GnRH immunostereilization vaccine capable of inhibiting or delaying sexual maturation, and with the potential to be more immunogenic than current GnRH vaccines. Recombinant hsp70 (DnAK) from M. tuberculosis was conjugated to the GnRH analogue GnRH-D6-Lys (with D-Lys substituted for glycine at position 6) using glutaraldehyde to link the molecules together via their lysine ε-amino and N-terminal groups (Avrameas et al. 1978). Prepubertal male mice were immunized with the GnRH-hsp70 construct in either a mild adjuvant, Ribi (an oil-in-water emulsion containing monophosphoryl lipid A (MPL) and synthetic trehalose dicorynomycolate (TDM)) (Todd et al. 1997), or as an emulsion in incomplete Freund’s adjuvant (IFA) with no active adjuvant. The effects of immunization on the reproductive system were evaluated by examining the histology of the testis and the gross morphology of the secondary reproductive organs, and quantitatively measuring changes in gene expression levels in the testis and the amount of serum testosterone.

Materials and Methods

Conjugation

GnRH-D6-Lys was conjugated to hsp70 using a glutaraldehyde coupling method (Avrameas et al. 1978), in which 0.7 mg GnRH-D6-Lys was mixed with 1.0 mg hsp70 in phosphate buffer (pH 5.0) in a final volume of 0.5 ml. Glutaraldehyde was added to a final concentration of 0.02% (v/v). The conjugation reaction was left at room temperature, shaking gently for 2 h. The reaction mixture was then dialyzed against PBS in a dialysis cassettes (Perbio, Tattenhall, UK) at 4°C overnight. The quality of the conjugation was analysed by SDS gel electrophoresis using a 10% polyacrylamide gel, followed by western blotting. Proteins were transferred onto a Hybond-C nitrocellulose membrane (Amersham), the membrane blocked with 5% (w/v) Marvel dried skimmed milk powder (Premier International Foods Ltd, Spalding, UK) in 0.1% (v/v) Tween 20 in PBS for 1 h at room temperature and then probed with a rabbit anti-GnRH antibody (Chemicon, Southampton, UK), diluted in 5% Marvel/0.1% Tween 20/PBS for 1 h. The membrane was then washed once for 15 min and twice for 5 min with 0.1% Tween 20/PBS and probed with a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Dako, Ely, UK) for 1 h at room temperature. The immobilized antibodies on the membrane were detected by ECL western blotting analysis system (Amersham). The membrane was exposed to Hyperfilm ECL (Amersham) for a few seconds and the film developed. The nitrocellulose membranes were stripped of antibody by adding 0.1 M glycine–HCl, pH 3.4. The membranes were left at 37°C for 30 min in the stripping solution, then washed as before prior to re-blocking. A macaque anti-hsp70 (kind gift of Dr L Bergmeier, King’s College London, UK) was used to re-probe the blot, and its binding detected with a goat anti-human IgG (Sigma) which cross-reacts with macaque IgG, followed by a swine anti-goat IgG HRP conjugate (The Binding Site, Birmingham, UK).
**Immunizations**

The immunogens were mixed with either Ribi MPL + TDM adjuvant or IFA (Sigma). Two milliliters of the GnRH conjugates were directly added to 10 ml of Ribi or to an equal volume of IFA while vortexing and then vortexed vigorously until a thick emulsion was formed. Male BALB/c mice, five per group in each of two separate experiments, were immunized with GnRH-d GS-lys-hsp70 conjugate (20 μg of GnRH per mouse) in either Ribi or IFA. Age-matched male BALB/c mice, five per group in each of two separate experiments, were immunized with PBS in Ribi as negative controls. The mice were injected i.p. with 200 μl at 3 weeks of age and given boosts at 5 and 8 weeks of age. They were killed 2 weeks after the last boost and blood collected by cardiac puncture. The mice were dissected, the testes weighed and one (randomly selected from the pair) was fixed in Bouin’s solution for histological analysis, the other flash frozen in liquid nitrogen for RNA analysis.

**ELISAs**

Ninety-six-well polypropylene ELISA plates (Maxisorp Nunc, Roskilde, Denmark) were coated with 50 μl per well of 10 μg/ml GnRH or hsp70 in 0.2 M carbonate–bicarbonate buffer (CBB) pH 9.6 overnight at 4°C, then blocked with 2% Marvel in PBS for 1 h at 37°C. The wells were washed three times with 0.05% Tween 20/PBS and incubated with diluted sera from the immunized mice in 0.05% Tween 20/PBS with 3% (w/v) BSA for 1 h at 37°C. The plates were washed as before and incubated with a rabbit anti-mouse-IgG alkaline phosphatase conjugate (Zymed, San Francisco, USA) for 1 h at 37°C. The ELISA plates were then washed three times with 0.05% Tween 20/PBS and once with CBB. The alkaline phosphatase substrate tablets (p-nitrophenyl phosphate, Sigma) were dissolved in CBB buffer according to manufacturer’s instructions and 50 μl added to each well. The color change was monitored at a wavelength of 405 nm in a MR5000 ELISA plate-reader (Dynatech, Billinghurst, UK). Each ELISA was carried out at least twice.

**Testosterone levels**

Testosterone assays were carried out with the radioimmunoassay Coat-A-Count Total Testosterone kit (Diagnostic Products Corporation, Los Angeles, USA) according to manufacturer’s instructions, with each measurement carried out in duplicate.

**Morphological analyses**

The urogenital complex, including the secondary reproductive organs such as the prostate and the seminiferous vesicles, was examined by a single assessor and graded for the degree of atrophy. Atrophied urogenital complexes were assigned a grade of 1 and fully mature complexes a grade of 3. Intermediates were given a grade of 2.

For histological analysis testis samples were fixed in Bouin’s solution (Sigma) overnight at 4°C. They were then washed in PBS for 30 min at 4°C, 1:1 (v/v) ethanol:PBS for 30 min at 4°C, 50% ethanol for 30 min at room temperature and then 75% ethanol followed by 100% ethanol both for 30 min at room temperature. The samples were then washed twice in toluene for 30 min, once in 1:1 (v/v) toluene:paraffin wax for 30 min at 65°C and twice in wax for 30 min at 65°C, before being embedded in paraffin wax. The samples were left at 4°C overnight and the paraffin embedded samples were then sectioned, the sections picked up onto polysine-coated microscope slides (VWR International, Poole, UK), left to dry overnight at room temperature and then baked at 37°C, again overnight. The sections were stained first with haemotoxylin stain (Sigma) and then counter-stained with eosin (Sigma) using standard procedures prior to mounting in DPX (Sigma). Slides were coded and evaluated blindly using a binocular microscope (Carl Zeiss, Welwyn Garden City, UK) with a 10 x 10 graticule in one of the eyepieces. The number of graticule cross-sectional points over the non-interstitial cells, lumen or interstitium were counted to evaluate the ratio of these different components. The counts were done on random sections of testis, in triplicate for each animal.

**Gene expression in the testis**

The relaxin-like factor (RLF) gene is expressed in the testis and, at much lower levels, in the ovary in adult mice (Zimmermann et al. 1997). Its expression is down-regulated in response to reduced testosterone production in the Leydig cells (Pusch et al. 1996). Determination of RLF mRNA levels was therefore used to evaluate the neutralizing effects of the GnRH immunizations and the effects of altered testosterone levels in the testis. Frozen testis, one from each mouse, and samples of negative control tissues (heart, liver and seminiferous vesicles known not to express RLF) from several of the mice were homogenized by ultrasonication in 3 ml of lysis solution (4.2 M guanidinium isothiocyanate, 1.3 mM sodium citrate, 10% (w/v) sodium lauryl sarcosinate, 5 mM 2-mercaptoethanol (7.2 μl/ml)). RNA was prepared using guanidinium isothiocyanate by standard procedures (Maniatis 1989). Forty micrograms of ethanol-precipitated RNA was resuspended in 25 μl of diethylpyrocarbonate (DEPC)-treated water and mixed with 49 μl of deionised formamide, 16 μl of formaldehyde and 10 μl of MOPS buffer (0.1 M 3-[N-morpholine] propanesulphonic acid, 5 mM EDTA, 0.5 mM sodium acetate, pH 7.0). The RNA samples were heated at 65°C for 5 min, placed on ice for 5 min, and then 100 μl of ice-cold 20 X SSC buffer (3 M NaCl, 300 mM Na 3C6H 5O) added. The RNA from each mouse was separately blotted onto Hybond-N (Amersham) nylon membrane in duplicate 50 μl samples using a commercial slot-blotter (BioRad, Hemel Hempstead, UK)
and the wells washed out with 10 x SSC buffer. The RNA was then covalently linked to the membrane by UV crosslinking.

The RNA blots were pre-hybridized in hybridization solution (7% SDS, 0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 0.1 μg/ml yeast RNA, 1% BSA) at 65 °C for at least 1 h and then probed with a relaxin-like factor (RLF)-specific cDNA (Pusch et al. 1996). The cDNA probe was labelled with 32P using the Rediprime II Labelling System (Amersham Biosciences) according to the manufacturer's instructions before adding to the membrane in the hybridization solution and incubation at 65 °C overnight. The membrane was washed in 2 x SSC, 0.1% SDS solution twice for 10 min at 65 °C and then exposed to a phosphorimager screen and analysed in a Storm phosphorimager (Amersham Biosciences) using ImageQuant software (Amersham Biosciences). The membrane was stripped of the radioactive probe with 50 ml of boiling 0.5% SDS solution and then allowed to cool down to room temperature. The membrane was washed briefly in 2 x SSC before being re-probed for detection of β-actin mRNA using the pTri-actin-mouse probe (Ambion, Huntingdon, UK). The intensity of the RLF signal was divided by the β-actin signal for each sample.

**Statistical analysis**

Statistical tests used were Student’s t-test, F-test (variance ratio test) and Pearson correlation coefficient test. Where the data did not conform to a normal distribution the non-parametric Mann–Whitney rank sum test was used.

**Results**

**GnRH-specific antibody responses**

GnRH was successfully covalently coupled to hsp70 as determined by comigration of the GnRH and hsp70 on SDS-PAGE and subsequent detection by specific antibody probing of a western blot (data not shown). The GnRH-specific antibody titres in serum samples from GnRH-hsp70-immunized mice and controls were determined by ELISA. An absorbance equal to or greater than the mean +3S.D. of the control group (mice immunized with PBS) was considered positive. All the GnRH-immunized animals showed an anti-GnRH response with no overall difference in the titres between the mice immunized with GnRH-hsp70/Ribi or with GnRH-hsp70/IFA (Fig. 1).

**Testosterone levels**

Serum testosterone was measured by radioimmunoassay. In the GnRH-hsp70/Ribi immunized mice, testosterone levels ranged from 0.16–0.30 nmol/l in nine of the mice, with one mouse having a testosterone level of 11.54 nmol/l. In the PBS/Ribi controls, testosterone levels ranged from 2.37–55.00 nmol/l. Thus, there was a significant reduction of testosterone levels in mice immunized with GnRH-hsp70/Ribi (mean 1.316 nmol/l) compared with mice immunized with PBS/Ribi (mean 20.852 nmol/l) ($P < 0.001$, Fig. 2a). The mice immunized with GnRH-hsp70/IFA did not show a statistically significant reduction in testosterone levels (data not shown).

**Morphology of the accessory reproductive organs and testis**

Accessory reproductive organs, such as the urogenital complex, showed marked atrophy in nine of the ten GnRH-hsp70/Ribi immunized mice ($P < 0.0005$, Figs 2b and 3). The testes were dissected from each animal (five per group/experiment) in the study. Testis weight did not differ significantly between the GnRH-immunized animals (0.196 ± 0.028 g, mean ± s.e.m., $n = 10$) and controls (0.218 ± 0.014 g, $n = 10$). One testis (randomly selected) was examined histologically and the other lysed and the
RNA isolated for gene expression analysis. The state of spermatogenesis is the most indicative factor of intact fertility. In all the GnRH-immunized mice there were detectable mature spermatozoa in the lumens of seminiferous tubules. There were, however, subtle changes in the morphology of the testis, as assessed by examining three different testis sections from each animal. In the GnRH-hsp70/Ribi-immunized mice the mean point scores ratio of non-interstitial cells: lumen: interstitium was 1: 0.141: 0.047, whereas for control mice immunized with PBS/Ribi the ratio was 1: 0.202: 0.170 indicating that the Leydig cell volume was significantly reduced in the GnRH-immunized mice (P, 0.005). In addition, increased numbers of meiotic round spermatids were detectable in the lumen of the seminiferous tubules in the GnRH-immunized animals (P < 0.05, Fig. 4).

Relaxin-like factor expression in testis

Expression of the testosterone-dependent RLF gene was significantly lower in the GnRH-hsp70 immunized mice (P ≤ 0.0005) compared with the control mice immunized with PBS/Ribi (Fig. 5).

Discussion

All of the GnRH-hsp70 immunized animals consistently developed GnRH-specific antibody responses irrespective of whether the vaccine was given with Ribi adjuvant or as a simple emulsion using IFA lacking microbial adjuvants. Although testosterone levels were significantly reduced in the groups immunized with GnRH-hsp70/Ribi, tests weights were not significantly different in immunized mice compared with control mice. However, the biological effects of the anti-GnRH antibodies elicited by the vaccine were clearly evident in the changes in testis morphology and in the gross atrophy of secondary reproductive organs making up the urogenital complexes. The more dramatic reduction in size of these organs compared with that of the testis might be explained by a higher proportion of androgen-dependent cells in the seminiferous vesicles and the prostate compared with LH-dependent cells in the testis. More subtle changes could be seen by looking at the histology and composition of the different compartments of the testis. The Leydig cells, together with blood vessels and testosterone-controlled interstitial fluid (Damber et al. 1992, Sharpe et al. 1994), constitute the interstitial compartment. There was a significant reduction in the interstitium in the GnRH-immunized animals. RLF expression, which is strongly dependent on testosterone levels, was significantly down-regulated in immunized animals.

In the presence of testosterone, around stages VII and VIII of the spermatogenic cycle, a tight association between the spermatid and Sertoli cell develops, possibly as a result of regulated synthesis of cell adhesion molecules (O’Donnell et al. 1996). However, testosterone withdrawal has been shown to result in the detachment of round spermatids from the seminiferous epithelium in rats (O’Donnell et al. 1996). It is therefore noteworthy that in mice immunized with GnRH-hsp70, there was an increase in the number of round spermatids in the meiotic stages of the spermatogenesis cycle released into the seminiferous vesicle lumen.

The serum testosterone levels were not reduced in every immunized mouse, despite the production of GnRH-specific antibodies in all of the immunized mice, indicating that the antibody-mediated neutralization of GnRH was not complete. This was particularly the case in the mice immunized with GnRH-hsp70/IFA. The reason for the incomplete neutralization of the peptide hormone could be that the quantity of GnRH-specific antibody or its affinity were not high enough, especially when the...
vaccine was given simply as an emulsion in the absence of microbial adjuvant. Alternatively, non-neutralizing antibodies may have been produced which prolonged the half-life of the peptide, making it more efficient at activating its receptor. GnRH neutralization affects testosterone levels by reducing LH levels (Ferro 2002). If GnRH neutralization was incomplete, the degree of reduction of LH levels might result in only a partial inhibition of Leydig cell production and testosterone secretion. The abrupt changes in serum testosterone levels seen during pubertal development, and the unclear relationship between local and systemic levels of testosterone, make this differential inhibition (for example between the gross atrophy of urogenital complex compared with the largely normal production of mature spermatozoa) rather difficult to dissect.

Presensitization with, or repeated usage of, a given carrier can induce ‘carrier suppression’ of the antibody response to the hapten conjugated to it (Renjifo et al. 1998). Hyporesponsiveness to GnRH conjugated to carriers in mice preimmunized with the carriers was found to be carrier-dependent and strain-dependent (Sad et al. 1991). It is of interest that the GnRH-immunized mice did not show any hsp70-specific antibody responses, although their GnRH-specific antibody responses were high, implying that all or most of the B cell epitopes on hsp70 were masked by the conjugated hapten. There would therefore presumably be little risk of carrier suppression in the secondary response to such a vaccine. Hsp70 is both an effective carrier and a powerful immunostimulant (Lehner et al. 2000). This results in high titre GnRH-specific antibody in mice immunized with GnRH-hsp70 without additional active adjuvant, i.e. in IFA which creates an antigen depot but does not itself contain microbial components to activate antigen-presenting dendritic cells.
In conclusion, we have developed a novel GnRH vaccine based upon covalent linkage of the peptide hormone to mycobacterial hsp70. The vaccine elicited GnRH-specific IgG antibodies in all the immunized animals. The presence of these antibodies was associated with substantial atrophy of the urogenital complex, significantly reduced amounts of testosterone-dependent testicular relaxin-like factor mRNA and significantly reduced serum testosterone levels. Thus, hsp70 might be a useful carrier for vaccines based upon reproductive hormones.

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