IMMUNOSTERILISATION AFFECTING THE FUNCTIONAL LEVEL OF REPRODUCTIVE HORMONES

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Abstract

Immunosterilisation in males is aimed at inhibiting the production of sperm by eliciting antibodies capable of neutralising the hormones that control spermatogenesis, gonadotrophin-releasing hormone (GnRH), luteinising hormone (LH), follicle-stimulating hormone (FSH) and testosterone. This approach is attractive as an alternative to surgical procedures for companion animals and animals bred for food purposes. Traditional castration can be time consuming and have a risk of infection and mortality. Additionally, immunosterilisation can improve meat and carcass characteristics in cattle, sheep, goats and boars, improve feed efficiency relative to castrates and reduce male aggressive behaviour and male-associated odours. Although immunosterilisation is unlikely to be used for fertility control in humans, there is great interest in using active immunisation against GnRH as means of treating steroid-dependent pathologies such as prostate cancer.

The work described in this thesis explores a number of design strategies aimed at producing an improved GnRH-based vaccine for male animals that would cause effective and irreversible sterilisation. Preliminary studies investigating the feasibility of increasing the efficacy of a GnRH vaccine by additionally neutralising another component in the hormonal reproductive pathway, LH, are also presented.

GnRH or a GnRH-analogue (GnRH-D6-Lys) were chemically coupled to the carrier molecules tetanus toxoid (TT) and heat shock protein 70 (Hsp70). Alternatively, they were expressed on the surface of bacteriophage particles or synthesised as part of a multiple antigen peptide (MAP) system along with a T helper cell epitope. These different carrier-antigen complexes in either Ribi adjuvant or without active adjuvant (in Freund's incomplete adjuvant) were used to immunise groups of male Balb/c mice repeatedly from 3 weeks of age to 12 weeks of age. Hormone-specific antibodies and serum testosterone levels were measured. In addition, the testes and the accessory reproductive organs were analysed for histological and morphological changes. The level of the testosterone-dependent relaxin-like factor (RLF) mRNA in the testes was also determined.
All the groups immunised with GnRH or ovine LH (oLH) conjugates mounted a hormone-specific antibody response, albeit at levels which varied between groups and between individual animals. Animals immunised with GnRH coupled to Hsp70 produced the highest level of specific antibodies. Although serum testosterone levels also varied, signs of testosterone reduction were apparent in histological analysis of the testes, involution of the seminiferous vesicles and prostate gland, and in the RLF expression in the testes following immunisations with most GnRH conjugates.

We hypothesised that targeting more than one component of the hormonal pathway (e.g. GnRH and LH) would achieve a more effective immunosterilisation than seen in experiments following immunisation with either one of the hormones alone. We did not, however, see such an additive effect in animals immunised with both GnRH constructs and oLH. Although the animals mounted an appreciable GnRH response, they produced very little oLH-specific IgG compared to mice immunised with oLH alone. These observations are discussed in relation to antigenic competition, carrier determinants and adjuvanticity.
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Abbreviations

aa Amino acid
Ab Antibody
Ala Alanine
Amp Ampicillin
APC Antigen-presenting cell
Arg Arginine
Asn Asparagine
Asp Aspartic acid
CD Cluster of differentiation
CG Chorionic gonadotrophin
Cys Cysteine
ddH2O Double-distilled water
DNA Deoxyribonucleic acid
dsDNA Double-stranded DNA
DTT Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
E.coli Escherichia coli
ECL Enhanced chemiluminescence
EDC 1-Ethyl-3-[3-Dimethylaminopropyl]carbodiimide Hydrochloride
eSG Equine serum globulin
FCA Freund’s complete adjuvant
FSH Follicle-stimulating hormone
Gln Glutamine
Glu Glutamic acid
Gly Glycine
GnRH Gonadotrophin-releasing hormone
hCG Human chorionic gonadotrophin
His Histidine
HRP Horseradish peroxidase
hSA Human serum albumin
hSG Human serum globulin
ICAM Intercellular adhesion molecule
IFN Interferon
Ig Immunoglobulin
IL Interleukin
Ileu Isoleucine
IPTG Isopropyl-β-D-thiogalactopyranoside
KAc Potassium acetate
kb kilobases
KLH Keyhole limpet haemocyanin
LB Luria Bertoni
LFA Lymphocyte function associated molecule
LH Luteinising hormone
LHRH Luteinising hormone releasing hormone
LiCl Lithium chloride
Lys          Lysine
LSHTM       London School of Hygiene and Tropical Medicine
MAP          Multiple antigen peptide
MCP          Membrane cofactor protein
MDP          Muramyl dipeptide
Met          Methionine
MHC          Major histocompatibility complex
MIP          Membrane inhibitory peptide
MIS          Müllerian inhibiting substance
NaCl         Sodium chloride
NaI          Sodium iodide
NaOH         Sodium hydroxide
P. aeruginosa Pseudomonas aeruginosa
PBS          Phosphate buffered saline
PEG          Polyethylene glycol
PFU          Plaque forming unit
Phe          Phenylalanine
PPD          Purified protein derivative
Pro          Proline
PVP          Polyvinylpyrrolidone
RIA          Radioimmunoassay
RNA          Ribonucleic acid
RNase         Ribonuclease
Rpm          Revolutions per minute
Ser          Serine
Spermatog    Spermatogenesis
ssDNA        Single-stranded DNA
TAE          Tris-acetate-EDTA
TBE          Tris-borate-EDTA
Tc           Cytotoxic T cell
Th           Helper T cell
Thr          Threonine
TNF          Tumour necrosis factor
Tris         Tris(hydroxymethyl)aminoethane (Tris base)
Trp          Tryptophan
TSH          Thyroid stimulating hormone
TT           Tetanus toxoid
Tyr          Tyrosine
Val          Valine
VCAM         Vascular cell adhesion molecule
VLA          Very late antigen
WHO          World Health Organisation
X-Gal        5-bromo-4-chloro-3-indolyl-β-D-galactoside
Chapter 1  General Introduction
1.1 Introduction

Reproduction and sexual behaviour in mammals is governed by various hormonal, neurological and social factors. In farm animals it has been necessary for centuries to castrate male animals because of problems relating to aggressive behaviour and to improve meat quality. Traditional sterilisation procedures are often considered to be inhumane, they are laborious and can cause infections and mortality (e.g. Gaynor et al., 1999, Robertson et al., 1994). Various alternatives to surgical procedures have been proposed, among them the use of a specific immunosterilisation 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.

The first section of this chapter will concentrate on the reproductive and immune systems and the immune responses to a vaccine. The second section discusses the possibilities of utilising vaccination to affect fertility. The third and fourth sections describe two hormones of the reproductive hormone pathway, gonadotrophin-releasing hormone (GnRH) and luteinising hormone (LH) and their potential roles in immunosterilisation vaccines. Lastly the fifth section discusses cascade inhibition and its applicability to an immunosterilisation vaccine.

1.2 The immune response to vaccination

An antigen is a molecule that is capable of being recognised by cells of the immune system. An immunogen is a molecule that is not only recognised by the immune system but also elicits an adaptive immune response. In the design of a vaccine against a particular antigen it is highly important to recognise which branch of the adaptive immune response, humoral or cell-mediated, is required to elicit a successful immune response against it. Figure 1.1 shows a schematic overview of the humoral and cell-mediated immune responses.
Figure 1.1  
Overview of the humoral and cell-mediated branches of the immune system

Secreted antibody binds to antigen and facilitates its clearance from the body. In the cell-mediated response various T cell subpopulations recognise antigen presented on self-cells. T\textsubscript{H} cells respond to antigen by producing cytokines. Precursor T cells respond by developing into cytotoxic lymphocytes (CTL) which mediate killing of altered self-cells (e.g. virus infected).
The humoral response

After vaccination extracellular antigen is taken up by antigen-presenting cells (APC) by either receptor-mediated endocytosis via complement receptors (CD35) or immunoglobulin (Ig) receptors (CD32) on their surface or by fluid phase endocytosis. APC are mainly found in the skin, lymph nodes, spleen, in mucosal epithelia and in the thymus. They can be B cells, macrophages or dendritic cells, e.g. the Langerhans' cells in the skin. The APC migrate to lymph nodes via the lymph system, to local lymphoid tissue, such as the mucosal-associated lymphoid tissue of the respiratory tract or intestine, or to the spleen, depending on the site of entry of the antigen into the body.

In the APC the antigen is digested into 10-24 amino acid long peptide fragments and presented on the surface of the APC in conjunction with a class II major histocompatibility complex (MHC). The MHCII/peptide complex can interact with specific T cell receptors (TCR) on the surface of CD4+ T helper (T_H) cells. The interaction is of low affinity and needs to be stabilised by various accessory adhesion molecules such as LFA-1/ICAM-1, CD2/LFA-3 and VLA-4/VCAM-1. Co-stimulation of the T cell by the co-stimulatory molecules B7-1/2 (CD80/CD86) on the APC and CD28 on the T cell results in the activation of the T cell (Jenkins et al., 1991). T cell activation results in increased gene transcription, importantly IL-2 transcription. Consequently the T cells start producing an array of other cytokines and cytokine receptors resulting in clonal proliferation of the T cell.

B cells can become activated either by thymus-independent antigens (either by polyclonal activation by B cell mitogens or by cross-linking of antibody molecules on the surface of the B cells) or by thymus-dependent antigens. Thymus-dependent antigens require T cell help to activate the naïve B cell into antibody production. Activation is initiated by the recognition of the processed antigen in a MHCII/peptide complex on the surface of a B cell by a TCR on a T_H cell and co-stimulated through interactions of CD40 with its ligand CD40L (CD154) (van Kooten and Banchereau, 1997).

The activated B cell starts expressing IL-4 receptor on its surface and IL-4 produced by the activated T_H cells stimulates the clonal proliferation of the B cells.
In the germinal centres of secondary follicles in the lymph nodes primed B cells proliferate in response to stimuli from the surrounding follicular dendritic cells (FDC), stimulated B cells and T cells (Chen et al., 1978, Grouard et al., 1996). The FDC bind antigen-antibody complexes via surface Fc receptors (CD32) and complement receptors (CD21 and CD35). The B cells die through apoptosis unless they are rescued (start expressing bcl-2) by either cross-linking of their slg molecules by the complexes attached to the FDC, activation of their CD40 receptors, which induces differentiation to memory B cells, or by soluble CD23 and IL-1α, which stimulate the cells to produce antibodies (Liu et al., 1991).

The B cell Ig genes undergo somatic hypermutation and only cells bearing receptors with high affinity for antigen are selected for survival (Berek et al., 1991, Jacob et al., 1991) resulting in affinity maturation of the produced antibody and a 100 to 10000 fold increase in affinity to the antigen. The B cells also undergo Ig class-switching influenced mainly by TH cells and their secreted cytokines, IL-4, IL-5, IL-6 and TNFβ (reviewed in Stavnezer, 1996).

**The cell mediated (cytotoxic) response**

Endogenous intracellular antigens such as viral proteins are cleaved by proteasomes and transported to the cell membrane in a complex with class I MHC molecules. The MHCI/peptide complex is presented on the surface of the cell to cytotoxic T cells. Activated cytotoxic T cells can induce apoptosis in their target cells by direct cell-cell signalling via their Fas ligands, indirectly by cytokine release (e.g. tumour necrosis factor β, TNFβ) or by exocytosis of cytoplasmic granules causing lesions in the target cell membrane.

Activated TH cells can be divided into two distinct groups (Mosmann and Coffman, 1989). TH1 cells produce cytokines such as interferon-γ (IFNγ), IL-2, TNFβ, TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF). They are effective stimulants of the cell-mediated immune response. TH2 cells on the other hand produce higher concentrations of IL-3, IL-4, IL-5, IL-6 and IL-10. They are effective helper cells for B cells. TH1 and TH2 responses are mutually antagonistic, i.e. TH1 cytokines inhibit proliferation of TH2 cytokine producing cells and vice versa (Seder and Paul, 1994) (see figure 1.2). This is important to have in mind when designing a vaccine.
and a vaccination protocol as the ratio of $T_{H1}$ to $T_{H2}$ response depends on the nature of the antigen, the dose, the adjuvant and the site of administration of a vaccine formulation (Roitt et al., 1998).

**B and T cell epitopes**

Antibodies usually recognise epitopes on immunogens in their native three-dimensional form. B cell epitopes can either be formed by amino acid residues that are sequential in the primary amino acid sequence (continuous) or that are far apart and are brought together by folding of the peptide chain into the native protein form (discontinuous). T cell receptors on the other hand recognise small linear peptide fragments of the antigen in association with MHC molecules. Additionally there is haplotype restriction of the T cell recognition of the MHC molecules, i.e. the T cells only respond to APCs bearing MHC molecules of the same haplotype as the host (Doherty and Zinkernagel, 1975).

Immunodominance of an epitope is when the immune response to it is stronger than to other epitopes on the same immunogen. Epitope competition can also occur between different epitopes within the same antigen or between different antigens. It may be due to density or accessibility of the epitope or affinity of the B cells’ surface Ig to the epitope. It may be due to the repertoire of the individual’s reactive B cells. Immunodominance can influence the efficacy of a specific vaccine. Vaccination can be ineffective when the secondary response to an antigen interferes with the primary response to a new antigen, a phenomenon called the ‘original antigen sin’ (Fazekas de St and Webster, 1966a, Fazekas de St and Webster, 1966b).

T cell epitope competition has been shown to be due to competition of antigenic peptides for binding to the MHC molecule on the APC and the antigen with highest affinity is called the dominant epitope (Ria et al., 1990). Identification of dominant epitopes on an immunogen is of importance in vaccine design as such epitopes could mask epitopes of the antigen that are targeted by the vaccine.
When Th cells have become activated they can be divided into two different subsets with distinct cytokine secretion patterns. IL-12, secreted by macrophages after intracellular infection, encourages a Th1 response. IL-4, possibly secreted by NK cells skews the response to Th2. The Th1 and Th2 cytokines are mutually inhibitory (red lines).

Th0 = Early helper T cell; NK = Natural killer; GM-CSF = Granulocyte-macrophage colony-stimulating factor; Mφ = Macrophage. (Based on picture in Roitt, 1997)
Epitope-specific vaccines are vaccines aimed at specific peptide sequences that constitute either a B or a T cell epitope. In this manner it may be possible to generate vaccines against subdominant or cryptic epitopes, i.e. epitopes that would not otherwise elicit a strong response, and prevent responses against undesirable epitopes.

1.3 The reproductive system

The reproductive hormone cascade is governed by a hypothalamic product, GnRH. The theory that the central nervous system is of critical importance in regulating reproductive cycles and seasonal changes in reproduction was first postulated by Harris and Green (Green and Harris, 1947). The isolation of GnRH and subsequent experiments on its biological activity showed that it is responsible for stimulating gonadotrophin secretion from the pituitary, and thus has a role in co-ordinating reproductive behaviour with environmental and seasonal changes (Schally et al., 1973). The gonadotrophins stimulate the gonads to produce sex steroids and control the synthesis of the gametes. Figure 1.3 shows schematically the hormonal cascade that governs the reproductive system in humans.
GnRH is produced in the hypothalamus. It stimulates the production and secretion of the gonadotrophins from the anterior pituitary. The gonadotrophins, LH and FSH stimulate steroidogenesis and gametogenesis in the gonads.

GnRH = Gonadotrophin-releasing hormone, LH = Luteinising hormone; FSH = Follicle stimulating hormone; hCG = Human chorionic gonadotrophin. (Based on picture in Talwar, 1997b)
1.4 Immunosterilisation

Farmed animals, including animals reared for food production, along with companion animals are routinely castrated for a number of reasons. In boars the meat quality is better if the animals are castrated. They then do not produce male hormones, which when broken down cause a strong odour of the fat. Surgery to sterilise larger animals such as horses and companion animals has a risk of infections and morbidity (Mee et al., 1998, Gaynor et al., 1999). As an alternative to surgical procedures sterilisation may be achieved by vaccination producing antibodies capable of neutralising the biological activity of components of the reproductive system. To be effective such a sterilisation vaccine would need to be reliable, convenient (i.e. less labourious than surgical procedures), cost efficient and safe. Several hormones have been targeted as antigens in immunosterilisation vaccines.

1.5 Breaking self-tolerance

The immune system does not generally make an antibody response against molecules that it considers as 'self'. An immunosterilisation vaccine aimed against reproductive hormones must therefore overcome the immunological tolerance to these self-molecules if an effective immune response is to be generated.

B and T cell tolerance is induced in the bone marrow and the thymus, respectively. T cells become tolerant to self-molecules by negative selection of developing T cells reactive to molecules in early life (Kappler et al., 1987, MacDonald et al., 1988). T cells can also become anergic if co-stimulatory activation signals are missing (Rammensee et al., 1989). This type of anergic tolerance can be induced outside of the thymus. Tolerance can fail to be induced against antigens that are anatomically isolated, such as proteins of the lens in the eye. This is also the case if molecules are restricted to organs that do not express MHC class II molecules or if there is only a low concentration of the processed molecule present on MHC class II molecules.
B cell tolerance is induced by negative selection or clonal deletion of cells that recognise self-molecules that are present in the bone marrow (Hartley et al., 1991, Nemazee and Burki, 1989). B cell tolerance can also be due to anergy, i.e. when a molecule is recognised by the surface Ig but lacks co-stimulatory signals from a helper T cell to activate the cell (Goodnow et al., 1988).

To effectively break the tolerance to a self-molecule the molecule can be linked to a carrier determinant that will provide strong helper T cell epitopes that can activate antigen-specific B cells (see figure 1.4). Because of the way B and T cells co-operate in the initiation of an immune response a poorly immunogenic peptide, such as GnRH, has to be physically linked to a carrier molecule ('linked-recognition'). Various modified, non-toxic, bacterial exotoxins such as tetanus toxoid (TT) and diphtheria toxoid (DT) have been used in such conjugate vaccine formulations. Large molecules such as keyhole limpet haemocyanin (KLH) and heat-shock protein 70 (Hsp70), amongst others, have also been used in experiments with carrier-conjugated immunogens.

Figure 1.4
'Linked recognition' of a hapten and a carrier molecule.

A carrier needs to be physically linked to a hapten (in this case a self-molecule) to induce T cell help. The primed B cell recognises an epitope on the hapten and can process and present carrier determinants to a $T_h$ cell.

A number of problems have been encountered on using carrier molecules. One is carrier-induced suppression of the antibody response. Presensitisation with a given
carrier, such as DT or TT, can induce hyporesponsiveness to the covalently linked antigen, in this case GnRH (Sad et al., 1991). This suppression (i.e. 'original antigen sin') is thought to be caused either by the action of suppressor T cells or by clonal dominance of carrier-specific B cells. Carrier-induced suppression can be circumvented by the use of synthetic T-helper epitopes instead of the whole carrier molecule (Sad et al., 1992).

The MHC genotype of an individual restricts the peptide repertoire that is recognised by the individual's T cells. This can be a problem in vaccine development, using T-helper epitopes, as T cell epitopes restricted to a certain MHC haplotype will not be recognised in individuals of a different MHC haplotype. Promiscuous T cell epitopes are peptide epitopes that are independent of MHC haplotype. These universal T cell epitopes are found for example in the malaria circumsporozoite protein, influenza haemaglutinin and in tetanus toxin. The peptide epitopes can be used for example in peptide-conjugate vaccines for outbred populations without haplotype restriction of the T cell response.

The response to a protein or peptide-conjugate antigen is also influenced by the dose administered, the solubility of the construct, the adjuvant used and the site of administration. The effective dose of antigen needs to be optimised for each antigen and species immunised. An adjuvant is any substance that has immunostimulatory properties. Adjuvants are usually not covalently linked to the antigen. The purpose of an adjuvant is twofold. Firstly to act as a depot of antigen, either by mixing it in various types of oils or by adsorbing the antigen onto large molecular weight particles such as alum to ensure slow release of the antigen. Secondly, adjuvants often contain bacteria or bacterial constituents, to enhance the immune response by stimulating the production of immunomodulatory cytokines and stimulating dendritic cells and macrophages to differentiate into APC. Different adjuvants promote different types of immune response; e.g. either Th	extsubscript{1} or Th	extsubscript{2} directed.

The route by which antigens are administered is important. Subcutaneous injections tend to elicit the strongest responses followed by intraperitoneal injections, while those injected directly into the blood stream often induce unresponsiveness and tolerance (Harlow and Lane, 1988). Antigens can also be presented by novel methods, such as in small lipid membrane vesicles (liposomes) or in immunostimulating complexes.
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(Iscoms). Liposomes can anchor an antigen in its lipid membrane and thereby induce a stronger cell-mediated immune response. Liposomes could also combine a number of factors such as carrier-conjugated peptide antigen, different adjuvants and targeting molecules, while Iscoms consist of adjuvant molecules, antigen, cholesterol and phosphatidylcholine. Biodegradable polymers can be used to produce controlled release vaccines, delivering an antigen over a period of up to several months, circumventing the need for multiple injections of an immunogen (e.g. Kissel et al., 1997, Gupta et al., 1996, Singh et al., 1998).

To affect the biological activity of a hormone such as GnRH it is necessary to produce enough neutralising GnRH-specific antibodies to completely neutralise the endogenous hormone. A strong humoral response is necessary for such high antibody titres and therefore a T_{H2} biased T cell response. This is achieved by choosing an adjuvant that favours T_{H2} rather than T_{H1} responses, conjugation of GnRH to a carrier that induces preferentially T_{H2} responses and/or immunisation in the presence of T_{H2}-inducing cytokines.

1.6 Immunosterilisation vaccines

Sterilisation is the process of making an individual unable to reproduce. It can be irreversible and is often used instead of the term castration, referring to removal of the testes. Contraception on the other hand refers to a means of preventing pregnancy and is usually reversible. Although the potential reversibility of immunosterilisation vaccines is sometimes discussed, the term is used here for a vaccine affecting the reproductive system irreversibly, unless otherwise stated. An immunocontraceptive vaccine is a vaccine designed to prevent contraception. ‘Anti-fertility vaccines’ can encompass both types of vaccines.

There is evidence of an immune response to seminal antigens, both in the male and in the female reproductive tract (Tung, 1978, D'Almeida and Voisin, 1979). Seminal antigens can be either within the acrosome or superficial. Secretions from the sexual accessory organs such as the prostate can also be antigenic. Sensitisation to these antigens in the male is usually prevented by the ‘blood-testes barrier’, made up by the
Sertoli cell and tight intercellular junctions (Hogarth, 1984). Spermatozoal antigens have been used in active immunisations and can induce infertility if given in appropriate adjuvant in a variety of species (reviewed in Hancock, 1984, Voisin, 1984).

The zona pellucida, an acellular layer formed around the oocyte, also contains potential antigens for an anti-fertility vaccine, such as ZP3 (Aitken et al., 1984).

The reproductive hormones are obvious candidates for anti-fertility vaccines since they either orchestrate the secretion of other reproductive hormones or control the production of gametes. Among the most extensively studied are GnRH, the gonadotrophins LH and follicle-stimulating hormone (FSH), and human chorionic gonadotrophin (hCG).

Various difficulties exist when making an anti-fertility vaccine against self-hormones such as the reproductive hormones. These will be discussed further in the following section.

1.6.1 Hormone based immunosterilisation vaccines

Problems associated with inducing an antibody response to self-molecules are numerous. Antigens might share determinants with other self-molecules and the antibodies against them could possibly cross-react. In the case of the reproductive hormones the glycoproteins have a large number of shared epitopes (Berger et al., 1996). Human CG for example has shared epitopes with LH and FSH and this is potentially dangerous for a vaccine targeted only at hCG as antibodies against hCG could cross-react with endogenous LH and FSH and interfere with the actions of these hormones. Epitope-specific vaccines offer a possible solution to this dilemma and vaccines have been synthesised that are specific to hCG epitopes only and antibodies are not cross-reactive with LH (Thanavala et al., 1980, Ramakrishnan et al., 1979). It is important to keep such considerations in mind when designing a vaccine especially when it is aimed at self-proteins.

Another concern is damage to tissues as a result of hypersensitivity responses, by antibodies generated against self-molecules. In successful immunisations against
GnRH in boars there were lesions in restricted parts of the hypothalamus of some of the immunised animals (Molenaar et al., 1993).

The effects of active immunisations of male animals against the reproductive hormones have in most cases been shown to be reversible in mature animals, normal fertility and androgen levels resuming when antibody levels fall below the threshold level needed for neutralisation of the hormones (e.g. Kumar et al., 2000). In immature animals neutralisation of the reproductive hormones can cause irreversible changes to the reproductive organs, that result in infertility in adult life.

1.6.2 Advantages and disadvantages of immunosterilisation vaccines

Immunosterilisation is an attractive alternative to surgical procedures, as it is easier to administer, less time-consuming and could be seen as a more humane method of castration. In animals bred for food purposes it is often desirable to postpone or prevent sexual maturation, such as in farmed salmon, in male pigs and bulls. Sterilisation vaccines could also be used on companion animals and as a pest control.

Sexual and aggressive behaviour of meat producing farm animals has led to animals being castrated at a very young age. However, research has found that intact animals have better feed efficiency and are leaner than castrated animals. Raising intact animals has other advantages such as lower production costs, reduction of environmental pollutants and improved animal welfare (Bonneau, 1998).

Besides the disadvantages of male behaviour, meat quality is also a determining factor in the decision to raise castrated instead of intact male animals. The main concern in raising intact boars is the presence of boar taint in the meat. Boar taint is mainly due to skatole, a product of tryptophane breakdown in the gut, and testicular steroids, such as androstenone in the fat (Bonneau, 1993). It is perceived as an unpleasant odour during cooking of the meat. Studies have shown that active immunisation of young male pigs against GnRH results in reduced testes development, reduced gonadotrophin (LH and FSH) and testosterone levels in serum, reduced androstenone levels and a reduced incident of boar taint (for a review see Bonneau and Enright, 1995).
GnRH is a prime candidate for an immunosterilisation vaccine. Previous immunisations have shown the efficacy of an immune response to GnRH in lowering gonadotrophin levels, testosterone levels and inhibiting spermatogenesis (e.g. Fraser and Gunn, 1973, Fraser et al., 1974, Bercu et al., 1977). There is, however, often a great deal of variance in response of the immunised animals. Also the traditional adjuvants that have been used, e.g. Freund's complete adjuvant, 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 animals tested and can be given in an acceptable adjuvant.

Immunosterilisation in males is directed at inhibiting the production of sperm and the hormones that control spermatogenesis, such as GnRH, LH, FSH and testosterone. The function of these hormones and their biological activity will now be discussed in more detail.

1.7 Gonadotrophin-Releasing Hormone (GnRH)

1.7.1 Structure and evolution of GnRH

GnRH is a single-chain decapeptide produced in the hypothalamus. Its amino acid sequence was first established in 1971 (Matsuo et al., 1971b, Matsuo et al., 1971a, Burgus et al., 1972) thereby confirming the existence of a hypothalamic factor stimulating the release of both LH and FSH from the anterior pituitary, as had been predicted as early as 1947 (Green and Harris, 1947). The sequence for mammalian GnRH is shown in figure 1.5.

The sequence of hypothalamic GnRH (GnRH-I) is highly conserved between mammalian species, but varies up to 50% between other vertebrate species (Kasten et al., 1996). Despite this variation its function as a regulator of reproduction has been conserved during 500 million years of evolution (Millar and King, 1988).
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**GnRH agonists:**
- Leuprolide: pGlu His Trp Ser Tyr DLeu Leu Arg Pro NH₂Et
- Buserelin: pGlu His Trp Ser Tyr DSer(O'Bu) Leu Arg Pro NH₂Et
- Nafarelin: pGlu His Trp Ser Tyr D2NalLeu Arg Pro Gly-NH₂
- Gosrelin: pGlu His Trp Ser Tyr DSer(O'Bu) Leu Arg Pro AzaglyNH₂
- Histrelin: pGlu His Trp Ser Tyr DHis(Bzl) Leu Arg Pro AzaglyNH₂
- Triptorelin pamoate: pGlu His Trp Ser Tyr DTrp Leu Arg Pro Gly-NH₂

**GnRH antagonists:**
- Nal-Glu Antagonist: Nal-Glu, ([Ac-D2Nal¹, D4ClPhe², D3Pal³, Arg⁵, DGlù⁶(AA), Al₅¹] GnRH)
- Cetrolix: [N-Ac-D-Nal(2)¹, D-Phe(pCl)², D-Pal(3)³, D-Cit⁴, D-Ala¹⁵] GnRH
- Ganirelix: [N-Ac-D-Nal(2)¹, D-pCl-Phe², D-Pal(3)³, D-Arg(Et)⁴(8), D-Ala¹⁵] GnRH
- Detirelix: [N-Ac-D-Nal(2)¹, D-pCl-Phe², D-Trp³, D-Arg(Et₂)⁴, D-Ala¹⁵] GnRH

**Figure 1.5**

**GnRH sequences**

a) The amino acid sequences of six GnRH isoforms. There are three known mammalian forms. The boxed parts of the sequences are the most invariant parts, i.e. the C- and N-termini. The N terminus is post-translationally modified to contain a pyro-glutamic acid and the C-terminus has been amidated.

b) GnRH agonist and antagonists
Some GnRH agonists that are currently available for clinical use and some GnRH antagonists that are undergoing clinical trials. GnRH agonists and antagonists are hoped to be of general use in treatment of prostate cancer in men and possibly also as a means of fertility control, given along with testosterone. (Based on figure in Yen, 1999)
It is now believed that there are at least three different forms of GnRH: GnRH-I, GnRH-II and GnRH-III (see figure 1.5). GnRH-I, the hypothalamic form, has a major role in reproductive regulation but its sequence varies greatly (up to 50%) between species (Sherwood et al., 1993). It is not only localised in the central nervous system, although it is in highest concentrations there, but also in the placenta, pancreas, gonads and some tumour cell lines (Sherwood et al., 1993). GnRH-II was first isolated from chicken brain and found to have His in position 6, Trp in position 7 and Tyr in position 8. (Miyamoto et al., 1984). It is invariant and has been found in such diverse species as fish, birds and mammals. GnRH-II is believed to be the most ancient and conserved form of GnRH (Millar and King, 1988).

The human gene encoding GnRH-II has recently been isolated and characterised (White et al., 1998). Both GnRH-I and II genes have four exons and encode a precursor containing signal sequences preceding the GnRH decapeptide, a three amino acid proteolytic site and a gonadotrophin-associated peptide (GAP, see below). This is the general organisation of the GnRH-I and GnRH-II genes in all other species analysed (Sherwood et al., 1993). The two GnRH precursors differ in size due to differences in the GAP sequence, which is 50% longer in GnRH-II than in GnRH-I (White et al., 1998). The main site for GnRH-II expression is in the extrahypothalamic areas of the midbrain (Chen et al., 1998) and it is also found in high levels in the kidney, bone marrow and prostate (White et al., 1998).

A third form of GnRH (GnRH-III) is found in the telencephalon of several fish species (Sherwood et al., 1983, Sherwood et al., 1984). A recent study showed that a related GnRH-III is expressed in the hypothalamic and midbrain neurones in mammals, including humans (Yahalom et al., 1999).

The biological functions of the different isoforms of GnRH are not yet clear. GnRH-II can act as a neuromodulator in spinal cord ganglion neurones in amphibia (Jan et al., 1980, Muske et al., 1994). Differentiated lymphocytes can also express GnRH or a GnRH-like peptide (Rissman et al., 1995, Marchetti et al., 1996). The diversity in their gene expression patterns would indicate that the GnRH peptides have diverse neurosecretory, endocrinological and even immunological roles.
All studied vertebrate GnRHs have an effect on the reproductive system, by stimulating gonadotrophin release. But the effects vary as there are considerable differences in the receptor binding and gonadotrophin-releasing activity of the different forms of GnRH (Millar et al., 1989, Millar et al., 1986). GnRH receptors have been found throughout the body, e.g. in the kidney and the prostate (Kakar and Jennes, 1995, Fekete et al., 1989). Recently a GnRH receptor specific for GnRH-II has been found in primates. It differs slightly in structure to the GnRH-I receptor and the two receptors are only 41% homologous in their nucleotide sequences, but they are both expressed ubiquitously in the body (Neill et al., 2001, Millar et al., 2001).

As shown in figure 1.5 the amino- and carboxyl-terminal ends of GnRH are highly conserved in vertebrates. Experiments with GnRH-analogues have shown that their N- and C-terminal sequences are involved in receptor binding and activation (Millar and King, 1988). GnRH has a bend in its middle region (Tyr\(^5\)-Gly\(^6\)-Leu\(^7\)-Arg\(^8\)) that brings the N- and C-termini close together in its active form (Momany, 1976, Lincoln, 1992). The N-terminus of mammalian GnRH is also protected by cyclization of the N-terminal glutamine to pyro-glutamic acid and the C-terminus is amidated (Lincoln, 1992, Pal et al., 1996).

Since GnRH-I is the predominant form of GnRH in the hypothalamus and the pituitary portal vessels (Chen et al., 1998) and is the most efficient of the known GnRH forms at stimulating LH release, in subsequent chapters 'GnRH' will refer to mammalian GnRH-I, unless otherwise stated.

**GnRH agonists and antagonists**

Numerous analogues of GnRH have been synthesised for possible therapeutic application. The responses to these GnRH-analogues have been evaluated by various different methods: LH secretion from pituitary cells in vitro, ovulation induction in vivo, oestrus suppression and inhibition of FSH-induced oestrogen production by granulosa cells in vitro (Hsueh et al., 1983). The analogues can be divided into agonist and antagonists. Agonists are more stable than the native form of GnRH and therefore have a longer half-life. They also have a higher receptor affinity, which along with the longer half-life results in increased biologic activity. Increased stability of the peptide is achieved for example by substitution at position 6 by a D-amino acid. Increased
receptor affinity is found by replacement of the C-terminal glycinamide residue with ethylamide. The interaction of GnRH with its receptor is discussed in detail in section 1.7.3. Some of these agonists are 200 times more efficient at stimulating the release of gonadotrophins from the pituitary than the native form of GnRH (Yen, 1999). The initial stimulation is however followed by down-regulation of the gonadotrophin-gonadal axis (see section 1.7.3).

Some synthetic antagonists on the other hand have higher affinity to the GnRH receptor than native GnRH, but do not evoke receptor activation. They are normally modified at the C- and N-termini or have deletions or substitutions at positions 2 and 3 (Karten and Rivier, 1986). A few agonists and antagonists of GnRH are listed in figure 1.5.

1.7.2 Biosynthesis of GnRH

GnRH is produced by neurosecretory cells in the hypothalamus. Immunocytochemical experiments have localised GnRH, GnRH-mRNA and GnRH precursor to a group of neurones that originate in various areas of the hypothalamus and terminate in proximity to portal capillaries in the median eminence, where GnRH is secreted into the portal vessels (Anthony et al., 1984, Standish et al., 1987). The portal vessels pass from the hypothalamus via the pituitary stalk (infundibulum) to the anterior pituitary. In the pituitary GnRH induces the synthesis and secretion of the gonadotrophins LH and FSH (Matsuo et al., 1971b). Figure 1.3 shows this hormone pathway schematically.

GnRH is produced as a 92 amino acid precursor protein (Seeburg and Adelman, 1984) (see figure 1.6). The first part of the precursor protein is a 23 amino acid long signal sequence. The signal sequence is cleaved off in the endoplasmic reticulum and the Golgi apparatus to expose a glutamine residue at the N-terminus of the GnRH sequence, that is either spontaneously or enzymatically cyclisised into pyro-glutamic acid (Burgus et al., 1972, Matsuo et al., 1971b). The precursor protein is also cleaved at the C-terminal end of the GnRH peptide, leaving a C-terminal glycine residue that is amidated (Bradbury et al., 1982).
The C-terminal 56 amino acids of the polypeptide are the gonadotrophin-associated peptide (GAP). GAP has independent hormonal functions. It can inhibit prolactin secretion and stimulate the secretion of LH and FSH although its efficacy is magnitudes lower than that of native GnRH (Nikolics et al., 1985). GnRH and GAP are transported through the endoplasmic reticulum, through the Golgi and secretory vesicles and co-secreted into the hypophyseal portal blood (Clarke et al., 1987, Silverman et al., 1990). GnRH is also synthesised outside the hypothalamus, most notably in the placenta. The precursor amino acid sequence shows only 70% homology between the rat and the human sequences, although the GnRH peptide itself is completely conserved between the species (Adelman et al., 1986). It is interesting to note that the hypogonadal mouse has a mutation that prevents the expression of the third and fourth exons of the GnRH gene, but the nucleotide sequence for the GnRH peptide itself is intact (Mason et al., 1986). Correct processing of the precursor is therefore necessary for GnRH expression, without which the reproductive system does not develop.

The prolactin-inhibitory effect of GAP requires the whole peptide, but the stimulatory effect on gonadotrophin release has been localised, by delineation of the peptide into peptide fragments, to the N-terminal region of the peptide, the minimal sequence being GAP_{4-13} (Milton et al., 1986, Wormald et al., 1989). A single amino acid substitution of D-Trp at position 9 of the peptide sequence GAP_{1-13} stimulates preferentially the release of FSH (Yu et al., 1990).

It can therefore be concluded that GAP plays an important role in regulating the production and secretion of the gonadotrophins and prolactin in the pituitary along with GnRH and that its functions could be greatly modified by differential cleavage before secretion to the pituitary.
Figure 1.6
The sequence of GnRH and its precursor

The gene structure and sequence of the human precursor protein for GnRH. Molecular weight of the precursor is 10,000 Da. The 23 amino acid signal sequence is followed by the GnRH decapeptide and the sequence (Gly-Lys-Arg) for enzymatic cleavage and C terminal amidation of GnRH. The remaining 56 amino acids constitutes the GAP sequence. GAP stimulates the secretion of LH and FSH and inhibits prolactin secretion.

(Based on picture in Johnson and Everitt, 2000)
1.7.3 Interaction of GnRH with its receptor

GnRH binds to highly specialised GnRH receptors on the pituitary gonadotrophs to induce a cascade of cell signalling events, that ultimately leads to stimulation of gonadotrophin secretion. Figure 1.7 summarises the signalling pathway following the binding of GnRH to its receptor.

The GnRH receptor is a 327 amino acid protein with seven transmembrane $\alpha$-helical domains. Analysis of the GnRH receptors primary amino acid sequence found it to be a member of the rhodopsin-like G protein coupled receptor (GPCR) family (Sealfon et al., 1997). Members of this diverse receptor family function to propagate a signal across a lipid membrane (Flanagan et al., 1999). The GnRH receptor lacks, however, the intracellular C-terminus that is typical for a GPCR and usually contributes to receptor desensitisation via phosphorylation. It is the smallest of this transmembrane receptor family, with closest homology to the receptor for IL-8. The GnRH receptor also differs from most GPCRs in that it has six potential phosphorylation sites on the three cytoplasmic loops, which could contribute to receptor desensitisation (Tsutsumi et al., 1992) (see figure 1.7).

GnRH receptors are initially evenly distributed on the cell surface but binding of GnRH induces dimerisation of the receptors and formation of clusters of receptors on the cell surface (Hazum et al., 1980, Conn et al., 1982). The bound GnRH and receptor are eventually internalised and a substantial number of hormone-receptor complexes are degraded in lysosomes although some of the GnRH receptors are recycled back to the cell surface. The dimerisation is necessary for activation of the receptor and the subsequent signalling cascade. The internalisation of the ligand-receptor complex is however not necessary for activation of the cell-signalling cascade as GnRH agonists, immobilised on beads, are capable of stimulating LH release even though the agonist-receptor complex is not internalised (Conn and Hazum, 1981). Some GnRH antagonists bind to GnRH receptors without inducing the dimerisation of the receptors, thus reducing gonadotrophin secretion (Hazum and Conn, 1988).
GnRH binds to the GnRH-receptor, which induces aggregation of the receptors and eventual internalisation. Activation of the GnRH-receptor results in activation of phospholipase C and protein kinase C. Ca2+ is released from the endoplasmic reticulum. Activation of protein kinase C results in protein phosphorylation and activation of the four groups of mitogen-activated protein kinase (MAPK) cascades, extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), p38MAPK and big MAPK (BMK). The activation of the kinases and their nuclear translocation affects the expression of the LH subunit genes and the secretion of LH by exocytosis from secretory vesicles. Phosphorylation sites on the cytoplasmic domains of the receptor may take part in the desensitisation of the receptor.

Based on figure in Naor et al, 2000.
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The ligand induced dimerisation of GnRH receptors activates the secondary messenger cascade via the activation of phospholipase C and generation of inositol-1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) (Snyder and Bleasdale, 1982, Andrews and Conn, 1986). They release calcium from intracellular sites and stimulate protein phosphorylation by activating protein kinase C (Jobin et al., 1995). Calcium influx is then involved in the secretion of LH by exocytosis from the cell (Tse et al., 1993, Tse et al., 1997). The GnRH receptors do not have a direct influence on the cAMP pathway, although there is evidence of synergistic action of the two pathways (Macrae et al., 1990).

The number of GnRH receptors on a cell is dependent on the pattern of secretion and the dose of GnRH. If GnRH pulses are given with either too short or too long intervals the number of receptors declines (Katt et al., 1985). The receptor recycling immediately after a GnRH pulse results in receptor up-regulation. The number of receptors on the cell surface decreases initially after a GnRH pulse but then increases to a higher value than before. Continuous exposure to GnRH or its agonists on the other hand maintains a state of receptor cross-linking (microaggregation) and results in suppression of gonadotrophin release and receptor down-regulation (Tsutsumi et al., 1992). The inhibitory action of GnRH agonists has been utilised in treatments of various disorders, such as prostatic hypertrophy, prostatic cancer, endometriosis and precocious puberty.

1.7.4 Biological functions of GnRH

GnRH is released from the hypothalamus into the pituitary portal blood vessels in a pulsatile manner (Knobil, 1980). This is evident from sampling of portal blood to measure GnRH and of peripheral blood to measure gonadotrophin levels (Knobil, 1980, Knobil, 1989, Clarke and Cummins, 1982). Each LH peak follows a peak in GnRH levels in the portal blood vessels, but each GnRH pulse does not necessarily evoke a peak in LH (Caraty and Locatelli, 1988). When GnRH is administered it invokes gonadotrophin secretion from secretory vesicles in the gonadotrophs within seconds. In addition secretory granules in the gonadotrophs are seen to mobilise to a region near the cell membrane and decrease in size (Nakamura and Yoshimura, 1986).
Consequently the next response to a GnRH pulse results in a larger (primed) LH release.

LH levels decrease at approximately the same rate as the hormone’s half-life, even if GnRH is administered in abnormally high quantities (Rasmussen and Malven, 1982). The amplitude of LH secretion seems to be independent of the amplitude of GnRH secretion. The frequency of the GnRH pulses however, does seem to be inversely related to the amplitude of LH secretion. Large peaks of LH levels have been detected after long intervals between pulses and smaller peaks after more frequent pulses (Lincoln, 1992). The magnitude of LH secretion is governed by the sensitivity of the pituitary gland for GnRH. Feedback mechanisms of gonadal steroids alter the effect of GnRH pulses on the pituitary and therefore the output of gonadotrophins. There is also evidence of an effect of endogenous GnRH on the pituitary gland, at levels that are not strong enough to elicit LH secretion, but might make the pituitary more susceptible to pulses of GnRH secretion (Lincoln et al., 1985).

GnRH and subsequent LH secretion is inhibited by the negative feedback action of gonadal steroids, such as progesterone, oestradiol and testosterone (see figure 1.9). Evidence of this action can be seen after castration or ovariectomy, when high peaks of LH secretion are detected (Lincoln and Short, 1980). High LH secretion can also be detected after immunisation of female animals against oestradiol, androstenedione and testosterone (Martensz et al., 1979, Martensz and Scaramuzzi, 1979, Ferin et al., 1974). Furthermore administration of progesterone and oestradiol either separately or together inhibit LH secretion in females and so does testosterone when given to castrated males (Martin et al., 1983, Lincoln, 1984).

In addition to the common factors controlling GnRH and LH secretion, GnRH and subsequent LH secretion in females is determined by a positive feedback loop in response to oestrogen release. Secretion of ovarian steroids, including oestrogen, changes during the reproductive hormone cycle, depending on the rate of follicle growth and the life span of the corpus luteum. It is well known that when oestradiol is administered in the absence of progesterone it normally evokes a pre-ovulatory LH surge (Barrell et al., 1992). Oestrogen inhibits GnRH secretion as the follicle matures. Oestrogen levels rise and eventually lead to a large surge of pulsatile GnRH secretion. Oestrogen greatly sensitises the responsiveness of the pituitary to GnRH (Reeves et al.,
1971, Drouin et al., 1976) and the GnRH surge results in greatly increased LH secretion from the pituitary. This LH surge results in ovulation. The corpus luteum then starts producing progesterone that, along with other factors, inhibits LH secretion (Lincoln, 1992).

GnRH synthesis and secretion starts during foetal life in mammals. GnRH has been found in the human foetus at 14 to 16 weeks (Kaplan et al., 1976). GnRH and gonadotrophin levels are frequently in the adult range soon after birth but decline and remain low during childhood until initiation of puberty. At puberty the hypothalamus starts producing more GnRH and releasing it in a pulsatile manner resulting in elevated levels of gonadotrophins and maturation of the gonads (Wildt et al., 1980, Ross et al., 1983).

Interestingly GnRH secretion can be influenced by neuronal factors as well as hormonal factors, as in lactational infertility that is induced by suckling (Lee et al., 1989) and coitus-induced ovulation (Short, 1967).

1.7.5 GnRH as an immunogen

Being the main regulator of gonadotrophin release from the pituitary, GnRH is the main target for an immunosterilisation vaccine. Small peptides, such as GnRH, are in general poor immunogens, often due to a lack of T cell epitopes. To overcome this they usually need to be coupled to larger carrier molecules and either adsorbed to high molecular weight substances (such as aluminium compounds) or supplemented with an adjuvant to evoke an immune response. In the case of GnRH, immunological tolerance to self must also be broken.

Carriers are often large proteins, such as KLH or TT, which contain T cell epitopes. The site of conjugation of a carrier to the GnRH peptide is important for the immunogenicity of the construct and the specificity of an antiserum raised against it. Conjugation to the middle region of the peptide results in antibodies raised against the N- and C- termini, and conjugation to either N- or C- terminus results in antibodies against the other end (Silversides et al., 1988).
General Introduction

**Active immunisation against GnRH**

Arimura and co-workers (Arimura et al., 1973) found that immunisation of rabbits with GnRH adsorbed onto polyvinylpyrrolidone in Freund's complete adjuvant resulted in production of GnRH neutralising antibodies which reduced pituitary LH content and showed atrophy of the testes. Similar responses were found in rabbits and rats immunised with GnRH conjugated to BSA when immunised in Freund's complete adjuvant (Fraser and Gunn, 1973, Fraser et al., 1974). These findings fuelled interest in research on GnRH immunogenicity in different species, with different carriers and adjuvants.

GnRH has in past experiments been coupled to numerous different carriers, including DT, TT, KLH, bovine serum albumin (BSA), human serum albumin (HSA), human serum globulin (HSG) and muramyl dipeptide (MDP) (e.g. Carelli et al., 1982, Fraser and Gunn, 1973, Goubau et al., 1989, Jayashankar et al., 1989, Ladd et al., 1990, Robertson et al., 1982). A list of some previous experiments on active immunisations with GnRH conjugates is in table 1.1. The table is not meant to be exhaustive, but to show the various formulations used to elicit an immune response against GnRH and the diversity of their effects. GnRH immunogens in various adjuvants have been used in a number of different species. The outcome of the immunisations is very heterogeneous and their effects on the reproductive system are not easily compared between experiments.

The efficacy of anti-GnRH vaccines is especially varied between different species of animal. To use a vaccine as a method of immunosterilisation it needs to be effective in all animals immunised, and preferably evoke a strong immune response following only one or two injections. To this end it has been proposed that tandem repeats of GnRH or other multivalent constructs of GnRH used along with a carrier molecule would be more efficient and show less variation than monomers of GnRH conjugated to carrier. Immunisations comparing the use of tandem repeats of GnRH-like peptides and monomers of GnRH-like peptide showed higher antibody response in some but not all animals immunised with the GnRH-tandem compared to the animals immunised with the monomer (Meloen et al., 1994).
<table>
<thead>
<tr>
<th>Species</th>
<th>GnRH conjugate</th>
<th>Adjuvant</th>
<th>Antibody response</th>
<th>Effect on LH</th>
<th>Effect on testes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>PVP</td>
<td>FCA</td>
<td>2/3 animals responded</td>
<td>reduced in responders</td>
<td>atrophy in responders</td>
<td>Arimura et al, 1973</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>FCA</td>
<td>3/3 animals responded</td>
<td>reduced</td>
<td>atrophy + no spermatog.</td>
<td>Fraser et al, 1973</td>
</tr>
<tr>
<td></td>
<td>BSA (C-term.)</td>
<td>FCA</td>
<td>varied</td>
<td></td>
<td>*</td>
<td>Nett et al, 1973</td>
</tr>
<tr>
<td>Rat</td>
<td>BSA</td>
<td>FCA</td>
<td>varied</td>
<td>reduced</td>
<td>atrophy + no spermatog.</td>
<td>Fraser et al, 1974</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>FCA</td>
<td>varied</td>
<td>reduced</td>
<td>*</td>
<td>Hauger et al, 1977</td>
</tr>
<tr>
<td></td>
<td>Thyroglobulin</td>
<td>FCA or alum</td>
<td>very varied</td>
<td>reduced</td>
<td>atrophy + no spermatog.</td>
<td>Bercu et al, 1977</td>
</tr>
<tr>
<td></td>
<td>DT</td>
<td>FCA</td>
<td>all</td>
<td>* (T reduced)</td>
<td>varied between groups atrophy</td>
<td>Morrison et al, 1987</td>
</tr>
<tr>
<td></td>
<td>TT (N-term., 5, 10)</td>
<td>Alum</td>
<td>varied</td>
<td></td>
<td>*</td>
<td>Jayashekar et al, 1989</td>
</tr>
<tr>
<td>Mouse</td>
<td>MDP or none</td>
<td>FCA or MDP</td>
<td>very varied</td>
<td>* (T reduced)</td>
<td>atrophy in responders</td>
<td>Carelli et al, 1982</td>
</tr>
<tr>
<td></td>
<td>MDP</td>
<td>FCA, FIA or PVP</td>
<td>varied</td>
<td></td>
<td>atrophy in responders</td>
<td>Carelli et al, 1985</td>
</tr>
<tr>
<td></td>
<td>Fimbriae</td>
<td>FIA</td>
<td>varied</td>
<td>*</td>
<td>atrophy</td>
<td>Mott et al, 1994</td>
</tr>
<tr>
<td>Dog</td>
<td>HSA</td>
<td>FCA</td>
<td>2/5 animals responded</td>
<td>reduced in responders</td>
<td>atrophy in responders</td>
<td>Schanbacher et al and English et al, 1983</td>
</tr>
<tr>
<td></td>
<td>TT (diff. conform.)</td>
<td>MDP</td>
<td>varied</td>
<td>* (T reduced)</td>
<td>variable atrophy</td>
<td>Ladd, 1994</td>
</tr>
<tr>
<td>Cat</td>
<td>TT (diff. conform.)</td>
<td>MDP</td>
<td>4/6 animals responded</td>
<td>* (T slightly reduced in some responders)</td>
<td>*</td>
<td>Ladd, 1994</td>
</tr>
<tr>
<td>Pig</td>
<td>α-globin</td>
<td>Mineral oil + saponin</td>
<td>very varied</td>
<td>reduced in responders</td>
<td>atrophy</td>
<td>Bonneau et al, 1993</td>
</tr>
<tr>
<td></td>
<td>HSG</td>
<td>FCA or MDP</td>
<td>all</td>
<td>reduced</td>
<td>atrophy</td>
<td>Falvo et al, 1986</td>
</tr>
<tr>
<td></td>
<td>KLH + tandem rep.</td>
<td>FCA</td>
<td>varied</td>
<td>* (T reduced in some)</td>
<td>atrophy in responders</td>
<td>Melloen et al, 1994</td>
</tr>
<tr>
<td>Sheep</td>
<td>BSA</td>
<td>FCA</td>
<td>varied</td>
<td>*</td>
<td>atrophy</td>
<td>Jeffcoate et al, 1982</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>FCA</td>
<td>varied</td>
<td>reduced</td>
<td>atrophy</td>
<td>Schanbacher, 1982</td>
</tr>
<tr>
<td></td>
<td>KLH, ESA, OVA, TT FCA</td>
<td>varied between groups</td>
<td>reduced</td>
<td>atrophy in 36/38 animals</td>
<td></td>
<td>Goubau et al, 1989</td>
</tr>
<tr>
<td>Cattle</td>
<td>HSA</td>
<td>FCA</td>
<td>5/10 animals responded</td>
<td>* (T reduced in some responders)</td>
<td>atrophy in responders</td>
<td>Robertson et al, 1979 and 1982</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>FCA</td>
<td>varied</td>
<td>*</td>
<td>*</td>
<td>Jeffcoate et al, 1982</td>
</tr>
<tr>
<td></td>
<td>Fimbriae</td>
<td>Oil-in-water</td>
<td>varied</td>
<td>*</td>
<td>varied between groups</td>
<td>Van der Zee et al, 1995</td>
</tr>
<tr>
<td>Horse</td>
<td>HSA</td>
<td>FCA</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Schanbacher and Pratt (unpublished)</td>
</tr>
<tr>
<td></td>
<td>OVA</td>
<td>Mineral oil + saponin</td>
<td>very varied</td>
<td>* (T reduced in some)</td>
<td>variable atrophy</td>
<td>Dowsett et al, 1993</td>
</tr>
<tr>
<td>Marmoset monkey</td>
<td>BSA</td>
<td>FCA</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Hodges and Hearn, 1977</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>BSA</td>
<td>FCA</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Chappel et al, 1980</td>
</tr>
<tr>
<td></td>
<td>DT</td>
<td>alum</td>
<td>varied</td>
<td>* (T reduced)</td>
<td>variable atrophy</td>
<td>Giri et al, 1991</td>
</tr>
</tbody>
</table>

Table 1.1

A few of the investigations into the effects of active immunisation against GnRH on the male reproductive system in mammals.

1 Different methods of detection of GnRH specific antibody titers were used in these experiments. In the table it is noted if the response showed considerable variance between individual animals or if some did not respond at all. *Not specified in reference.

FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant; PVP = polyvinylpyrrolidone; BSA = bovine serum albumin; PPD = purified protein derivative; DT = diphtheria toxoid; TT = tetanus toxoid; MDP = muramyl dipeptide; HSA = human serum albumin; HSG = human serum globulin; KLH = keyhole limpet hemocyanin; ESA = equine serum albumin; OVA = ovalbumin; T = testosterone.
General Introduction

Most of the immunisation experiments have been done in Freund's complete adjuvant and with repeated injections of the vaccines. The ulcerative properties of FCA make it unacceptable as an adjuvant in a vaccine for use in farm or companion animals (Stevens, 1993), and so it is necessary to find an alternative vaccine formulation that does not require administration in FCA. Falvo et al (Falvo et al., 1986) showed that muramyl dipeptide (MDP) can be used as an alternative to FCA and is equally effective if repeatedly administered. Bonneau et al (Bonneau et al., 1994) immunised boars with GnRH conjugated to α-globulin in mineral oil and then boosted with saponin as adjuvant. Androstenone levels in immunised animals were reduced drastically and the adjuvants used are acceptable for use in farm animals, but as in previous experiments the magnitude of the antibody response showed extensive heterogeneity within the group.

The biological effects of neutralising GnRH are varied. In male animals there is usually reduction of testosterone and LH levels in serum and marked atrophy of the testes and other sexual organs, such as the prostate and the seminiferous vesicles. Spermatogenesis is also arrested in animals with an efficient immune response (Arimura et al., 1973, Fraser et al., 1974). The mechanism of GnRH blockage is however not completely understood. There may be an agonistic effect of the GnRH construct for native GnRH. It could bind to receptors but not activate them, thus down-regulating them and switching off reproductive functions relating to GnRH. The GnRH producing cells could also suffer some damage from the vaccine, since the GnRH producing cells in the median eminence are not protected by the blood-brain barrier and are to some extent accessible to the immune system. Such damage has been found in immunisations with a tandem repeat of GnRH coupled to KLH carrier protein (Molenaar et al., 1993). This would be unacceptable in an immunosterilisation vaccine. Lastly the neutralising effect on GnRH could be due to the binding of GnRH-specific neutralising antibodies to GnRH in the pituitary portal blood, thereby hindering its binding to the GnRH receptor on pituitary cells. To support this explanation various immunisation experiments have shown that the effects on the reproductive systems of immunised animals are often directly proportional to the GnRH-specific antibody titres in the animals' sera (e.g. Arimura et al., 1973, Fraser et al., 1974, Meloen et al., 1994).
Immunocastration by active immunisation against GnRH is a viable alternative to surgical castration in farmed animals. In humans on the other hand immunisation against GnRH has been utilised as a cancer therapy. Immunisation with a GnRH vaccine with DT conjugated to GnRH with lysine at position 6 has undergone phase I and II clinical trials in males as a treatment for prostate cancer. It is found to reduce levels of testosterone and the size of the prostate in male rats (Jayashankar et al., 1989). In patients with advanced carcinoma of the prostate a rise in anti-GnRH antibodies was followed by a decrease in serum testosterone to castration levels and a reduction in prostate-specific antigen (PSA), a marker for disease of the prostate. Scans also showed a reduction in prostate mass (Talwar, 1997b).

**Passive immunisation against GnRH**

Passive immunisation against GnRH has the advantage of immediately and specifically neutralising GnRH. It is also more reproducible than active immunisation. Passive immunisation has been invaluable in determining the physiological properties of GnRH, but is not practical as a therapeutic tool or as a method of fertility control, as it would require vast amounts of antisera and multiple injections as the duration of the antibodies injected is relatively short.

### 1.8 Luteinising Hormone (LH)

Luteinising hormone (or lutropin) is a member of the glycoprotein family that includes FSH, thyroid-stimulating hormone (TSH) and CG (produced in the placenta of some species). The gonadotrophins LH, FSH and CG all have a function in controlling the production of gametes and sex steroids.

The members of the glycoprotein hormone family all share a similar structure. They are composed of two non-covalently linked subunits, $\alpha$ and $\beta$. The $\alpha$ subunit is common to all of the hormones but the $\beta$ subunit is species and hormone-specific and confers biological activity to each hormone (Pierce and Parsons, 1981). The $\alpha$ and $\beta$ subunits are found separately in the pituitary but do not have full endocrine activity unless as an $\alpha\beta$ heterodimer (Moyle and Campell, 1992).
1.8.1 Gene organisation and regulation of expression

A single gene encodes for the α subunit in mammals (Fiddes and Goodman, 1981, Fiddes and Talmadge, 1984). It is 9.4 kilobases (kb) long and is expressed in the pituitary for the synthesis of LH, FSH and TSH and in the placenta for CG. The α polypeptide has a molecular weight of approximately 14 kDa, however the native α subunit is glycosylated and has therefore a higher molecular weight. Comparison shows amino acid sequence homology of 74 percent between the rat and the human sequences and 95 percent between the rat and the mouse sequences (Godine et al., 1982). This high homology is consistent with the observation that an α subunit from one species can associate with a β subunit from another (Fiddes and Talmadge, 1984).

The β subunits of the glycoproteins share some sequence homology. The human LHβ and hCGβ sequences are highly homologous (82% homology of the amino acid sequences), except for the C-terminal extension found on hCG (Talmadge et al., 1984). The basic gene structure is the same in all the glycoprotein hormones: three exons divided by two introns. Separate genes on separate chromosomes encode for each β subunit within a species, with the exception of the gene for hCGβ. The hLHβ gene is in a complex LH/CGβ gene cluster consisting of one LHβ gene, 2 CGβ genes and 5 CGβ pseudogenes (Policastro et al., 1986, Talmadge et al., 1984). In humans, cows, sheep, rats and mice there is one LHβ gene that is 1.1 kb long (Jameson et al., 1984).

The α and β subunits both have mRNA encoding for leader peptides ensuring that the subunits are translocated to the lumen of the endoplasmic reticulum during synthesis. The signal peptide is cleaved off cotranslationally and oligosaccharides are attached to the relevant sites of glycosylation (asparagine residues) on both subunits. The partially glycosylated α and β subunits combine in the endoplasmic reticulum and the oligosaccharides undergo further modification in the Golgi complex en route to secretory granules, where the fully formed and glycosylated protein is stored until it is secreted (Halvorson and Chin, 1999). The β subunit contains invariant regions that are involved in subunit interactions, which explains how different β subunits can combine with identical α subunits (Halvorson and Chin, 1999).
X-ray crystallography studies have determined the three-dimensional structure of the hCG molecule. Because of their high sequence homology (82%) the structure of hCG can be used as a model for the three-dimensional structure of human LH. All four glycoprotein hormones (LH, CG, FSH and TSH) in humans have a conserved number of cysteine residues in the same positions on their subunits. There are five disulphide linkages in the \( \alpha \) subunit and six in the \( \beta \) subunit. The disulphide linkages form a structure called a cysteine knot, common to a family of hormones, including growth factors such as nerve growth factor and platelet-derived growth factor-\( \beta \) (Lapthorn et al., 1994). The \( \alpha \beta \) heterodimer is held together by noncovalent forces, but in addition a region of the \( \beta \) subunit wraps around the \( \alpha \) subunit and stabilised by a disulphide bond (Cys\(^{26}\)-Cys\(^{110}\) in hCG\( \beta \)). This is called the seat belt region of heterodimer (Lapthorn et al., 1994). Figure 1.8 shows the three-dimensional structure of hCG.

Both \( \alpha \) and \( \beta \) subunits are highly glycosylated and the sugar residues play an important role in the bioactivity and half-life of the hormones (Pierce and Parsons, 1981). The removal of the carbohydrate groups of LH\( \beta \) results in loss of biological activity, i.e. the ability to activate target cells, but does not affect binding to its receptor (Ryan et al., 1988). As the glycoprotein hormones have specific \( \beta \) subunits it would seem that the \( \beta \) subunits were the determining factor of the bioactivity of the hormone. Interestingly, when the subunits are deglycosylated and recombined, it is the sugar residues of the \( \alpha \) subunit not the \( \beta \) subunit that are critical for receptor activation of the hormones (Sairam, 1989). From these and other structural studies it can be deduced that there are regions of both the common \( \alpha \) subunit and of the specific \( \beta \) subunit that must interact with the hormone receptor to result in activation.

The main characteristics of the gonadotrophins in mammals (humans taken as example) are summarised in table 1.2.
LH and hCG show many structural similarities. They are both αβ heterodimers, with a common α subunit, held together by noncovalent forces. In addition a region of the β subunit wraps itself around the α subunit and is stabilised by a disulphide bond.
<table>
<thead>
<tr>
<th></th>
<th>LH</th>
<th>FSH</th>
<th>CG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secreted from</strong></td>
<td>Anterior pituitary gonadotrophs</td>
<td>Anterior pituitary gonadotrophs</td>
<td>Cytotrophoblast, then syncytiotrophoblast of placenta in pregnancy</td>
</tr>
<tr>
<td><strong>Acts upon</strong></td>
<td>Leydig cells</td>
<td>Sertoli cells</td>
<td>Luteal cells</td>
</tr>
<tr>
<td></td>
<td>Thecal cells – antral follicles</td>
<td>Granulosa cells – follicles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Granulosa cells – preovulatory follicles</td>
<td>Granulosa cells – follicles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Luteal cells – corpus luteum</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interstitial glands of the ovary</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
<td>approximately 28 kDa</td>
<td>approximately 33 kDa</td>
<td>approximately 37 kDa</td>
</tr>
<tr>
<td><strong>Composition</strong></td>
<td>α subunit – 92 aa</td>
<td>α subunit as in LH</td>
<td>α subunit as in LH</td>
</tr>
<tr>
<td></td>
<td>β subunit – 121 aa</td>
<td>β subunit – 111 aa</td>
<td>β subunit – 145 aa</td>
</tr>
<tr>
<td><strong>Receptor</strong></td>
<td>85-92 kDa glycoprotein; G protein coupled</td>
<td>146 kDa multimeric glycoprotein; G protein coupled</td>
<td>As for LH</td>
</tr>
</tbody>
</table>

**Table 1.2**  
The main characteristics of the human gonadotrophins.

kDa = Kilodalton  
(Based on table in Johnson and Everitt, 2000)
1.8.2 Regulation of LH synthesis and secretion

Gonadotrophin synthesis and secretion is regulated at many levels. Regulation is at a hypothalamic level, mainly by GnRH secretion, pituitary level, by the peptides activin, inhibin and follistatin and at a gonadal level, by steroidal feedback mechanisms.

Pulsatile GnRH secretion is discussed in section 1.7. In addition to influencing the amount of LH and FSH released, GnRH pulsatility also influences the ratio of LH to FSH release. Experiments with hypothalamic lesioned animals have shown that rapid GnRH pulses favour LH secretion whilst slower pulse frequencies favour FSH secretion (Haisenleder et al., 1991, Wildt et al., 1981).

GnRH clearly has an effect on the secretion of gonadotrophins, but it also has an effect on their synthesis. This is apparent by experiments done on cultured pituitary cells. Radiolabelled amino acids are incorporated into LH and FSH at an increased rate after GnRH is added to the cells (Khar et al., 1978). If mRNA synthesis is blocked the GnRH-induced LH synthesis is inhibited (Liu and Jackson, 1978). There is also evidence for increased gonadotrophin mRNA expression in response to GnRH administration both in vivo and in vitro (Wierman et al., 1989, Hamernik and Nett, 1988, Kaiser et al., 1997).

A few other neuropeptides besides GnRH have an effect on gonadotrophin release. Neuropeptide Y (NPY) is produced in the thyrotrophs in the anterior pituitary and stimulates GnRH release in the presence of sex steroids (Kalra and Crowley, 1992). Galanin and pituitary adenylate cyclase-activating polypeptide also have an effect on gonadotrophin release, although as with the NPY, their exact function is unknown. They might act indirectly on GnRH pulsatility or they might have a direct paracrine effect on the gonadotrophs (Rawlings and Hezareh, 1996, Lopez et al., 1991, Arimura, 1998).

Products of the gonads such as the gonadal steroids and the polypeptides activin, inhibin and follistatin all have some effect in the feedback mechanisms that govern gonadotrophin synthesis and secretion. After removal of the gonads (gonadectomy) there is a marked rise in plasma levels of the gonadotrophins, so the effect seems to be
mainly inhibitory (Gharib et al., 1987). The gonadal steroids include oestrogens, progesterones and androgens. The exact mechanism of sex steroid action in controlling gonadotrophin synthesis is unknown but it seems to act at the hypothalamic and pituitary levels. There are receptors for the various steroids on a number of neuronal cell types in the hypothalamus including those that secrete dopamine and β-endorphin, which have a direct effect on GnRH pulsatility (Romano et al., 1989, Sar, 1984). Interestingly sex steroid receptors have not been found on cells producing GnRH (Huang and Harlan, 1993). Gonadotroph cells in the anterior pituitary also have sex steroid receptors (Spranglers et al., 1989).

Oestrogen could in addition have a direct effect on GnRH synthesis and secretion as there are oestrogen-responsive DNA-regulatory regions within the GnRH gene promoter (Radovick et al., 1991). Oestrogen can be either stimulatory to GnRH release or inhibitory depending on reproductive state. Oestrogen has an inhibitory effect when given after gonadectomy on the rise in LH and FSH secretion (Shupnik et al., 1988). Conversely oestrogen has a positive feedback effect on the production of LH at the time of the midcycle surge of LH in females (Young and Jaffe, 1976).

Androgens can also be either stimulatory or inhibitory on gonadotrophin secretion. After gonadectomy in males, testosterone treatment inhibits the rise in LH synthesis and secretion. It does not however have an effect on FSH secretion (Gharib et al., 1987). Contrary to their inhibitory effect on LH synthesis and secretion at the hypothalamic level, androgens have a stimulatory effect on FSH synthesis in the pituitary in both sexes but little or no effect on LH synthesis (Gharib et al., 1990, Wierman and Wang, 1990).

Activin, inhibin and follistatin are all polypeptides originally isolated from follicular fluid. They have since been found in various tissues outside of the gonads. Inhibin decreases gonadotroph function, whereas activin stimulates it. Since these polypeptides are found to be expressed in various reproductive and non-reproductive tissues their functions are probably diverse and tissue-specific (Meunier et al., 1988).

Figure 1.9 summarises the factors that regulate gonadotrophin biosynthesis and secretion.
The gonadal steroids have an inhibitory effect on GnRH release at hypothalamic and pituitary levels. Activin, inhibin and follistatin exert their effects at both hypothalamic and pituitary levels. (Based on picture in Yen, 1999)
1.8.3 Biological function of LH

The main functions of the gonadotrophins in the gonads is to stimulate steroidogenesis and gametogenesis. Activation of the gonadotrophin receptors results in stimulation of the adenylate cyclase system that in turn stimulated steroidogenesis and gametogenesis.

The LH/CG-receptor

Gonadotrophin receptors belong to the membrane bound, G protein-coupled receptor family (McFarland et al., 1989). They are found in low concentrations but with high affinity and specificity on the target cell membrane (Yamoto et al., 1992). LH and CG use the same receptor, called the LH/CG receptor. The FSH receptor seems to share the main structural characteristics of the LH/CG receptor. The human LH/CG receptor is a single chain polypeptide 674 (ovary) and 669 (testis) amino acids long (Loosfelt et al., 1989, Minegishi et al., 1990). As is common for G-protein coupled receptors, it has seven transmembrane domains, with a relatively short intracellular C-terminus and a large extracellular N-terminal domain (McFarland et al., 1989). The receptor contains a number of phosphorylation sites that could partake in receptor activation and deactivation. The N-terminal extracellular domain contains the ligand-binding sequences and has 14 leucine-rich repeats that facilitate the interaction of the hydrophilic extracellular domain and the hydrophobic transmembrane domain, which results in the activation of the receptor (Krantz et al., 1991). The extracellular domain also contains six N-linked glycosylation sites, which are highly conserved between species, pointing to an important functional or structural role for the carbohydrates (Minegishi et al., 1989). Disruption of the glycosylation does not always affect hormone binding to the receptor but does influence the activation of the receptor (Liu et al., 1993). Ligand binding to the LH/CG receptor activates the cAMP mediated protein kinase A pathway via the G-protein $G_s$ as well as the protein kinase C pathway via the G-protein $G_q$ (Allgeier et al., 1994, Zhu et al., 1994). Figure 1.10 summarises the structure of the LH/CG receptor and the intracellular signalling pathways schematically.
Binding of LH causes changes in the secondary contacts of the hormone and the extracellular loop/transmembrane region that then initiate signal transduction.

In the Leydig cells the LH receptor is primarily coupled to $G_S$, leading to stimulation of the cAMP/PKA pathway. Stimulation of PI hydrolysis and stimulation of phospholipase C results from stimulation by either $G_q$ or more likely the $\gamma\beta$-subunits released from $G_S$ or $G_I$ (Dufau, 1998).
The gonadotrophin receptors are expressed on various gonadal and extragonadal tissues. LH/CG receptor is found on the granulosa, luteal, theca and interstitium cells of the ovary and the interstitium cells (Leydig cells) of the testes. It has also been found on the human endometrium, myometrium, fallopian tubes and in the brain (Ziecik et al., 1992, Sprengel et al., 1990, Camp et al., 1991). There are therefore clearly extragonadal functions associated with the gonadotrophins and their receptors. FSH receptors are found on the granulosa cells of the ovary and the Sertoli cells of the testes (Camp et al., 1991).

### Gonadotrophin control of the gonads

The control of the reproductive cycle by the gonadotrophins in females is a complex, multifactorial process and will not be discussed in detail here. There are many parallels between the neuroendocrine control of gonadal activity in the sexes. The main difference is that in the male steroidogenesis and gametogenesis occur continuously and not cyclically as in the female. Seasonal breeders are an exception as spermatogenesis and androgen output show seasonal variations throughout the year.

The testes have two major functions: sex steroid production and spermatogenesis. Spermatogenesis is under strict control both spatially and temporally in the testes. Sertoli cells seem to be the controlling cells of the process since they form a continuous cytoplasmic network that provides an environment in which the spermatogenic cells can develop. Leydig cells produce the major androgen of the testes, testosterone. Other gonadal factors produced by the Leydig cells are oestrogens, oxytocin and the polypeptide relaxin-like factor (RLF). When LH binds to its receptor intracellular cAMP levels in the Leydig cell rise within a minute followed by a rise in testosterone 20-30 minutes later (Dufau et al., 1984). Prolactin and inhibin also have an effect on testosterone production in the Leydig cells along with LH, but neither hormone has a stimulatory effect on testosterone production on its own.

Testosterone is mainly released into the peripheral blood stream and the lymph, by which it is carried to accessory sex glands, which are stimulated by it. Testosterone is lipid soluble and can therefore pass through the blood-testes barrier to enter the seminiferous tubules. Sertoli cells have androgen receptors on their surface and
derivatives of testosterone can bind to and activate the receptors stimulating gametogenesis.

### 1.8.4 Immunisation with LH

The heterogeneity of the βLH subunit has been utilised in interspecies immunisations. Both passive and active immunisations have been done in a variety of species. Immunisation of males against LH affects LH-dependent androgen production in the Leydig cells. The efficacy of the immunisation is measured by its effect on the fertility of the animal, the size and weight of the testes and other accessory organs, testosterone levels in blood and histological changes in the testes, including changes in size and appearance of the Leydig cells and development of spermatogenesis (e.g. Wakabayashi and Tamaoki, 1966, Quadri et al., 1966). Table 1.3 summarises some of the experiments done by active immunisation against LH.

Early experiments done on rabbits, raising antibodies to purified sheep LH (ovine LH, oLH), showed that the antisera cross-reacted with pituitary preparations of a number of different species (Moudgal and Li, 1961). Rats passively immunised with anti-oLH sera showed signs of androgen deficiency, i.e. reduced size of the testes and accessory organs (Hayashida, 1963, Moudgal and Li, 1961).

Active immunisations of rats and rabbits with oLH in Freund’s complete adjuvant (FCA) also resulted in marked degenerative effects on the reproductive system. Although the antibodies cross-reacted with oFSH and oTSH specific effects due to the cross-reactivity were not observed (Quadri et al., 1966, Wakabayashi and Tamaoki, 1966). Effects on the testes show extensive variation between different experiments both in rats and in rabbits. An interesting phenomenon was recorded after immunisation of rabbits against bovine LH (bLH). There seemed to be thickening of the basement membranes of the tubules and proliferation of Leydig cells, suggesting an enhanced action of LH rather than reduced (Pineda et al., 1967). This contrasts with other observations of LH immunised animals. An explanation might be that after successful immunisation against LH androgen production in the testes is impaired and this lifts the androgen induced inhibition of LH synthesis in the pituitary and results in increased rather than reduced LH levels as seen after surgical castration.
<table>
<thead>
<tr>
<th>Species</th>
<th>LH type or LH conjugate</th>
<th>Adjuvant</th>
<th>Antibody response</th>
<th>Affect on T</th>
<th>Effect on testes/ accessory sex glands</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>oLH</td>
<td>FCA</td>
<td>all animals responded</td>
<td>reduced</td>
<td>atrophy and reduced weights</td>
<td>Wakabayashi and Tamaoki, 1965</td>
</tr>
<tr>
<td></td>
<td>oLH</td>
<td>none</td>
<td>no response</td>
<td>no effect</td>
<td>no effect</td>
<td>Wakabayashi and Tamaoki, 1965</td>
</tr>
<tr>
<td></td>
<td>oLH</td>
<td>FCA</td>
<td>*</td>
<td>reduced</td>
<td>reduced weight and azoospermic</td>
<td>Awoniyi et al, 1989</td>
</tr>
<tr>
<td>Rat</td>
<td>oLH</td>
<td>FCA</td>
<td>all animals responded</td>
<td>reduced</td>
<td>atrophy and reduced weights</td>
<td>Wakabayashi and Tamaoki, 1965</td>
</tr>
<tr>
<td>Rabbit</td>
<td>oLH</td>
<td>FCA</td>
<td>all animals responded</td>
<td>reduced</td>
<td>reduced weight and azoospermic</td>
<td>Jeyakumar et al, 1995</td>
</tr>
<tr>
<td></td>
<td>oLH</td>
<td>FCA</td>
<td>all animals responded</td>
<td>reduced</td>
<td>reduced weight and azoospermic</td>
<td>Pineda et al, 1967</td>
</tr>
<tr>
<td>Dog</td>
<td>pLH</td>
<td>FCA</td>
<td>varied</td>
<td>reduced in responders</td>
<td>reduced weight in responders</td>
<td>Lunnen et al, 1974</td>
</tr>
<tr>
<td>Pig</td>
<td>oLH</td>
<td>FCA</td>
<td>very varied</td>
<td>no effect</td>
<td>no effect</td>
<td>Falvo et al, 1986</td>
</tr>
<tr>
<td></td>
<td>pLH-hSG</td>
<td>FCA</td>
<td>varied</td>
<td>no effect</td>
<td>no effect</td>
<td>Falvo et al, 1986</td>
</tr>
<tr>
<td>Sheep</td>
<td>pLH</td>
<td>FCA</td>
<td>dependent on amount of pLH used</td>
<td>reduced</td>
<td>reduced weight</td>
<td>Fahmy et al, 1999</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>oLHβ</td>
<td>FCA</td>
<td>response (1/1)</td>
<td>*</td>
<td>*</td>
<td>Moudgal et al, 1985</td>
</tr>
</tbody>
</table>

Table 1.3
Some experiments looking at the affects of active immunisation against LH on the male reproductive system in mammals.

1It is noted if the response showed considerable variance between individual animals or if some did not respond at all.

*Not specified in reference.

oLH = ovine LH; bLH = bovine LH; FCA = Freund's complete adjuvant; hSG = human serum globulin; T = testosterone.
The outcome of immunisation against LH depends on the age of the animals when they are first immunised, whether they have reached puberty, are immature or mature adults (for a review see Wickings and Nieschlag, 1984). Passive immunisation using anti-LH antibodies in neonatal rats resulted in infertility for the remainder of their lives (Goldman et al., 1971). The effects of passive immunisations on adult animals were reversible. Rats passively immunised with anti-oLH antibodies showed severe androgen deficiency and subsequently a reduction in testes weight, atrophy of accessory organs such as the seminiferous vesicles, arrest of spermatogenesis and various histological changes (Hayashida, 1963). Active immunisation against LH shows similar changes in the reproductive system as after passive immunisation (Wakabayashi and Tamaoki, 1966). Testosterone levels in either passively or actively immunised rabbits were reduced, in some cases to castration levels. Libido and normal androgen-dependent behavioural patterns could be restored by testosterone replacement in immunised animals, although testosterone on its own was not always enough to restore normal spermatogenesis (Quadri et al., 1966).

The other gonadotrophins, FSH and hCG, have also been targeted as possible candidates for an anti-fertility vaccine, inducing a reversible change in the reproductive system for possible use in humans. Immunisation against FSH results in degenerative changes to the reproductive system similar to those seen after LH immunisations, although not to the same extent. Passive immunisation of immature rats with anti-FSH antibodies resulted in a decrease in testis weight, but had no effect on accessory glands and testosterone levels (Madhwa Raj and Dym, 1976). Results from active immunisations with FSH and passive immunisations with anti-FSH antibodies are very diverse, but in most cases some degenerative effects on spermatogenesis are found in immunised animals. Studies on monkeys have revealed that neutralisation of FSH by passive immunisation with anti-FSH antibodies causes reduced spermatogenesis due to regression of the germinal epithelium, proving that FSH is required for normal spermatogenesis (Wickings et al., 1980).

Human CG is produced by the placenta starting at the blastocyst stage of the embryo. Immunisation against hCG has therefore been viewed as a potential anti-fertility vaccine for women and extensive research has been done in that field, leading to clinical trials of three of the vaccines (reviewed in e.g. Talwar, 1997b). A major
drawback in using hCG, LH or FSH in an anti-fertility vaccine is the cross-reactivity found between antisera to one glycoprotein hormone with another hormone. The glycoprotein hormones all share various characteristics, besides having identical $\alpha$ subunits. The $\beta$ subunits on their own, or fragments of them, have therefore been used instead of the whole hormone.

Successful immunisation against LH results invariably in reduced androgen levels. Androgens affect not only spermatogenesis but also libido and other male behaviour patterns. LH is therefore a prime candidate for an anti-fertility vaccine for use in male animals.

1.9 Cascade inhibition

Previous vaccines targeting GnRH have had very varied results. Considerable individual variation between immunised animals hinders objective analysis of the efficacy of the vaccines. Experiments using LH have also yielded diverse results, varying greatly between different species. The potentials and shortcomings of an immunosterilisation vaccine against GnRH or LH in male animals have already been discussed. There is a need for an immunosterilisation vaccine that uses an acceptable adjuvant, requires a limited number of injections, is efficient and consistent.

Vaccine development is often hampered by low immunogenicity of the antigens used and the inconsistent antibody responses generated against the antigen. Cross-reactivity of the antisera is also a major problem. Epitope-specific vaccines offer the possibility to generate a completely specific immune response, e.g. specifically against LH with no cross-reactivity with other glycoproteins. Multicomponent and multivalent vaccines have been shown to be more effective in various vaccine trials. Development of an effective malaria vaccine for example has recently been focused on multicomponent vaccines, which include antigens specific for different stages of the parasite’s life cycle. These multistage vaccines have proven to induce immune responses that inhibit the parasite’s development at multiple stages, thereby giving better protection against the disease (Shi et al., 1999).
GnRH and LH represent subsequent stages in the reproductive hormone cascade, one stimulating the release of the other. Neutralisation of these consecutive steps could have a multiplied effect on the outcome of the pathway, in this case testosterone release and spermatogenesis (see figure 1.11). Neutralisation of GnRH has a major effect on the reproductive pathway, as it controls the synthesis and secretion of both FSH and LH. As LH stimulates the release of testosterone from the testes its neutralisation should result in a dramatic decrease in circulating testosterone levels. Reduction of androgen levels is one of the main goals in the development of an immunosterilisation vaccine directed at farm animals.

![Figure 1.11](cascade_inhibition_of_the_reproductive_pathway.png)

Cascade inhibition of the reproductive pathway.

GnRH and LH are consecutive steps in the reproductive hormone pathway. Neutralisation of GnRH by x % and LH by y % should result in a multiplied effect on the outcome of the pathway, i.e. steroidogenesis and gametogenesis in the testes.
Aim

The aim is to define strategies for improved immunosterilisation vaccine formulations and to test the hypothesis that an immunosterilisation vaccine using cascade inhibition of the reproductive pathway is more efficient than a vaccine against single stages of the pathway. GnRH and LH have been chosen as candidates for the vaccine as they are central in the control of spermatogenesis and synthesis of androgens. The efficacy of various different constructs of GnRH immunogens to affect spermatogenesis and reduce testosterone levels is also compared.

An improved immunosterilisation vaccine for male animals, causing effective and irreversible sterilisation, could have widespread use in farm animals, companion animals and even as a pest control.
Chapter 2  Materials and Methods
2.1 Laboratory reagents

Laboratory chemicals and reagents were obtained from various sources. A list of suppliers follows.

2.1.1 Suppliers

<table>
<thead>
<tr>
<th>Materials</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bisacrylamide (30%)</td>
<td>Amresco</td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide (Gene-Page 6%)</td>
<td>Amresco</td>
</tr>
<tr>
<td>Agar</td>
<td>Gibco BRL (Life Technologies)</td>
</tr>
<tr>
<td>Agarose</td>
<td>Bio/Gene Ltd</td>
</tr>
<tr>
<td>Alkaline phosphate substrate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Antibodies:</td>
<td></td>
</tr>
<tr>
<td>Rabbit anti GnRH</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Goat anti rat LH</td>
<td>Insight (Santa Cruz Biotech.)</td>
</tr>
<tr>
<td>Rabbit anti hCG</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit anti M13</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Macaque anti-Hsp70</td>
<td>Kindly given to us by L. Bergmeier (King's College London)</td>
</tr>
<tr>
<td>Rabbit anti goat IgG-Alk Phos</td>
<td>Sigma</td>
</tr>
<tr>
<td>Swine anti goat IgG-Peroxidase</td>
<td>The Binding Site</td>
</tr>
<tr>
<td>Goat anti human IgG</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit anti mouse IgG1-Alk Phos</td>
<td>Zymed</td>
</tr>
<tr>
<td>Rabbit anti mouse IgG2a-Alk Phos</td>
<td>Zymed</td>
</tr>
<tr>
<td>Rabbit anti mouse IgG2b-Alk Phos</td>
<td>Zymed</td>
</tr>
<tr>
<td>Rabbit anti mouse IgG3-Alk Phos</td>
<td>Zymed</td>
</tr>
<tr>
<td>Rabbit anti mouse IgA-Alk Phos</td>
<td>Zymed</td>
</tr>
<tr>
<td>Rabbit anti mouse IgM-Alk Phos</td>
<td>Southern Biotechnology</td>
</tr>
<tr>
<td>Goat anti mouse IgG-Peroxidase</td>
<td>Sigma</td>
</tr>
<tr>
<td>Goat anti rabbit IgG-Alk Phos</td>
<td>Sigma</td>
</tr>
<tr>
<td>Goat anti rabbit IgG-Peroxidase</td>
<td>DAKO Ltd</td>
</tr>
<tr>
<td>Bacto-tryptone</td>
<td>Bio/Gene Ltd</td>
</tr>
<tr>
<td>Bacto-yeast extract</td>
<td>Bio/Gene Ltd</td>
</tr>
<tr>
<td>Boric acid</td>
<td>BDH</td>
</tr>
<tr>
<td>Bouin’s solution (fixative)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma</td>
</tr>
<tr>
<td>CaCl₂ (calcium chloride)</td>
<td>BDH</td>
</tr>
<tr>
<td>Chroma spin + TE 30 column</td>
<td>Clontech Laboratories, Inc</td>
</tr>
<tr>
<td>Cryotubes (1.5-2.0 ml)</td>
<td>Nunc</td>
</tr>
</tbody>
</table>
Materials and Methods

DEPC
DMMO tissue culture medium
DTT (dithiothreitol)
ECL
EDTA
EDC
ELISA plates (96 well, flat bottom plates)
1.5 ml ‘Eppendorf’ tubes
0.5 ml ‘Eppendorf’ tubes
Ethanol (99.7-100%)
15 ml ‘Falcon’ tubes
50 ml ‘Falcon’ tubes
Foetal calf serum (FCS)
Formamide
Formaldehyde
Freund’s incomplete adjuvant (F5506)
Glacial acetic acid
Glutaraldehyde (25%)
Glycerol
Glycine
GnRH human
GnRH-D6-Lys
GnRH-MAP
GnRH-Th-MAP

Helper-phage (R408)
Hsp70

Hybond membranes (C, N and N+)
IPTG
Isoamylalcohol
Isopropanol (Propan-2-ol)
KAc (potassium acetate)
KCl (potassium chloride)
KH2PO4 (potassium phosphate)
KLH (H7017)
L-Glutamine
LH (bLH, hLH, oLH and rat LH)
MES
Microscope film (Ektachrome 64T)
Miniprep kits
NaCl (sodium chloride)
Na2HCO3 (anhydrous sodium carbonate)
NaH2CO3 (sodium bicarbonate)
Na2HPO4 (sodium phosphate)
NaOH (sodium hydroxide) pellets
PD-10 columns (Sephadex G-25)
Penicillin/Streptomycin
Pfu polymerase

Sigma
Gibco BRL (Life Technologies)
Sigma
Amersham Pharmacia Biotech
Sigma
Pierce & Warriner (Perbio)
Nunc
Greiner Labortechnik Ltd
Greiner Labortechnik Ltd
BDH
Greiner Labortechnik Ltd
Greiner Labortechnik Ltd
Gibco BRL (Life Technologies)
Sigma
Sigma
Sigma
BDH
Sigma
Sigma
Sigma
Sigma
Sigma
Sigma
Sigma
Sigma
Sigma
Sigma-Genosys
Kindly given to us by Dr. K.S. Iyer (Inst. for Research in Reproduction, Mumbai, India)
Stratagene
Kindly given to us by Dr. R. van der Zee (Utrecht University)
Hybaid
Sigma
Sigma
BDH
BDH
BDH
BDH
Sigma
Gibco BRL (Life Technologies)
NHPP, NIH, NIDDK
Sigma
Kodak (Sigma)
Hybaid, Nucleon and Qiagen
Sigma
BDH
BDH
BDH
Amersham Pharmacia Biotech
Gibco BRL (Life Technologies)
Promega
Materials and Methods

Pipes
200 μl plastic tips
1000 μl plastic tips
Rainbow marker
RbCl (rubidium chloride)
Rediprime II random labelling system

Restriction enzymes and corresponding buffer:
BglII + buffer
BamHI + buffer
EcoRI + buffer
XbaI + buffer
Ribi- MPL+TDM adjuvant system
Ribi- MPL+TDM+CWS adjuvant system
SDS
Sequenase kit (sequencing reactions)
Temed
Tetanus toxoid
T-helper peptide
Tissue culture flasks and plates
Tissue culture pipettes
Tris
Trypsin solution
Tween
X-gal

Buffers and other solutions were made according to Sambrook (Sambrook et al., 1989), unless otherwise stated. All buffers used are listed in Appendix B.
2.3 DNA protocols

2.3.1 Cloning methods

Restriction endonuclease digests, polynucleotide kinase reactions, alkaline phosphatase reactions and ligations were essentially as described in enzyme manufacturer's instructions.

2.3.2 Bacterial culture

Bacterial strains used were *E. coli* strains:

**JS5** (F- araD139 Δ(ara-leu)7696 Δ(lac)X74 galK hsdR2 (rk-mk-) mcrB1 rpsL (str^R))

**JM101** (SupE thi Δ(lac-proAB) F' [tra D36 proAB^ lac') lacZ ΔM15])

They were grown in Luria Bertoni (LB) medium containing 75 μg/ml ampicillin or LB plates containing 2% agar and 75 μg/ml ampicillin or in 2xYT medium.

Wherever possible blue/white selection was performed on transformed colonies on ampicillin plates coated with 100 μl of 100 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) and 20 μl of 50 mg/ml X-gal. The pBluescript (SK+) plasmid (Stratagene) contains the gene for the α fragment of β-galactosidase (β-gal) with the multiple cloning site within it. The host *E. coli* strains lack the gene for the α fragment so α complementation following transformation by pBluescript and expression of its α fragment gene can yield functional β gal. The colonies are stained blue if the agar plate contains the inducer for β gal expression IPTG and X-gal (chromogenic substrate). The presence of an insert in the α fragment gene in pBluescript disrupts the α fragment gene expression and those colonies remain white.
2.3.3 Preparation of competent cells

A single colony of JS5 or JM101 *E. coli* was grown in 5 ml LB medium overnight in a shaking incubator at 37°C and then used to inoculate 200 ml of LB medium. The culture was incubated at 37°C, shaking at 200 rpm until it reached an OD$_{550}$ of 0.4. Cells were chilled on ice for 5 minutes and then harvested by centrifugation at 2500 x g for 15 minutes at 4°C in pre-chilled sterile 50 ml tubes. The pellet of cells was then resuspended in 40 ml of ice-cold buffer I (see appendix B) and incubated on ice for 5 minutes. The cells were then centrifuged again at 3000 x g for 5 minutes at 4°C and the resulting pellet resuspended in 2 ml of ice-cold buffer II. The cells were aliquotted in 50 µl aliquots into pre-chilled 1.5 ml cryotubes after 15 minutes on ice and then flash frozen in liquid nitrogen. The competent cells were stored at –80°C.

2.3.4 Transformation of competent cells

50 µl of freshly thawed cells were mixed with 5 µl of 10 µg/ml plasmid DNA and chilled on ice for 45 minutes. A negative untransformed control (no added DNA) was always included. The cells were heat shocked at 42°C for 2 minutes and then cooled on ice for 1-2 minutes. After adding 1 ml LB medium the cells were left shaking at 37°C for 1 hour before the cells were pelleted, resuspended in 100 µl of LB medium and plated onto LB agar plates containing the appropriate antibiotic (ampicillin). The plates were incubated at 37°C overnight.

2.3.5 Purification of plasmid DNA

Small scale preparations of plasmid DNA (mini-preps)

5 ml of LB medium, containing the appropriate antibiotic (75 µg/ml ampicillin), was inoculated with a single bacterial colony and incubated in a shaking incubator at 37°C overnight. 1.5 ml of culture was poured into an Eppendorf tube and the cells harvested by centrifugation at 10,000 x g for 10 seconds, resuspended in 100 µl of cold solution I (see Appendix B) and left for 5 minutes at 4°C. 200 µl of solution II was added and left for 5 minutes at 4°C to lyse the cells before 150 µl of solution III was added at 4°C.
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for 5 minutes, to precipitate genomic DNA and cellular proteins and neutralise the solution. The samples were centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was extracted with an equal volume of phenol:chloroform and precipitated in 2 volumes of 100% ethanol at -20°C for 30 minutes. The plasmid DNA was collected by centrifugation at 10,000 x g for 15 minutes at 4°C, washed with 70% ethanol and resuspended in 50 μl TE buffer (pH 8.0) or distilled water, containing 20 μg/ml of RNase. (Sambrook et al., 1989)

Alternatively mini-preps were prepared using Nucleon DNA kits or Qiagen DNA preparation kits according to manufacturers’ protocols. The Nucleon miniprep protocol uses a DNA binding resin. The cells were harvested, lysed by alkaline lysis and the cellular debris precipitated. The lysate was added to the DNA binding resin, vortexed and centrifuged at 14,000 x g for 30 seconds. The resin was washed with 500 μl of wash buffer, allowed to dry for 5 minutes at 60°C and then eluted in 50 μl of TE buffer (pH 8.0). The Qiagen DNA preparation kit protocol uses spin columns (QIAprep columns) with a silica-gel membrane to adsorb plasmid DNA. The bacterial cells were harvested, lysed and subsequently neutralised and adjusted to high-salt binding conditions. The lysates were centrifuged at 14,000 x g for 10 minutes and the supernatant added to a QIAprep spin column and centrifuged at 14,000 x g for 1 minute. The column was washed in 750 μl of wash buffer and centrifuged twice at 14,000 x g for 1 minute. The plasmid DNA was eluted in 50 μl of TE buffer (pH 8.0).

Large scale preparations of plasmid DNA (maxi-preps)

200-500 ml of LB medium were inoculated with 5 ml of bacterial culture, grown from one colony overnight, and incubated at 37°C for 10-15 hours in a 2.5 litre flask. The bacteria were harvested by centrifugation at 3000 x g for 5 minutes at 4°C and resuspended in 10 ml of ice cold solution I. The cells were lysed by adding 20 ml of solution II, neutralised with 15 ml of solution III and centrifuged at 5000 x g for 15 minutes at 4°C. The supernatant was filtered through four layers of cheesecloth before the DNA was precipitated with 1 volume of isopropanol at room temperature for 10 minutes and recovered by centrifugation at 5000 x g for 15 minutes. The pellet was washed with 70% ethanol, allowed to air-dry and then dissolved in 3 ml of TE (pH 8.0).
Materials and Methods

Plasmid DNA was purified further by polyethylene glycol (PEG) precipitation. Excess RNA was removed by adding 3 ml of ice-cold 5M LiCl and centrifuging at 10,000 x g for 10 minutes at 4°C. The plasmid DNA was precipitated with an equal volume of isopropanol and recovered by centrifugation at 5000 x g for 15 minutes, washed with 70% ethanol and allowed to air-dry. The pellet was dissolved in 500 µl TE (pH 8.0) containing 20 µg/ml of RNase and left at room temperature for 20 minutes. 500 µl of 1.6 M NaCl and 13% (w/v) PEG was added and the plasmid DNA was recovered by centrifugation at 10,000 x g for 5 minutes and then dissolved in 400 µl TE (pH 8.0). The excess PEG was removed by extraction once with phenol, once with phenol:chloroform (1:1) and once with chloroform. The aqueous phase was transferred to a fresh tube and 100 µl of 10 M ammonium acetate added, followed by 2 volumes of ethanol. After 10 minutes at room temperature the DNA was recovered by centrifugation at 10,000 x g for 5 minutes, washed with 70% ethanol, the pellet air-dried and then resuspended in 500 µl of TE (pH 8.0).

The amount of DNA was determined by measuring the absorbance at 260 nm (OD_{260}) of a 1:200 dilution (1 OD_{260} = 50 µg of plasmid DNA/ml). The prep was aliquoted and stored at -20°C. (Sambrook et al., 1989)

2.3.6 Agarose gel electrophoresis

Agarose was dissolved in 1 x TAE buffer at concentrations of 0.8-2.5% depending on the size of the DNA fragments to be separated. Ethidium bromide (1 µg/ml) was added before the gel was cast. The DNA samples were mixed with 1 x DNA loading buffer, spun down briefly and loaded onto the agarose gel. The DNA fragments were separated at 80-100 volts for 1-2 hours in 1 x TAE buffer.

DNA fragments were recovered from agarose gels by excising the DNA band and solubilising the gel in 2 volumes of 6 M NaI at 55°C. An appropriate amount of DNA-binding matrix (silica suspension: 100 mg/ml of silica (Sigma) in 3 M NaI), usually 15 µl, was added and mixed thoroughly. The mixture was centrifuged at 10,000 x g for 5 minutes at room temperature. The resulting pellet was washed twice with 500 µl of wash buffer (Appendix B). The DNA was eluted in 30 µl of TE (pH 8.0) or distilled water.
Alternatively a DNA purification kit from Hybaid was used. A gel slice (not more than 300 mg) was added to 400 µl of Binding Buffer (containing a DNA binding silica gel) in a spin filter tube, heated to 55°C for 5 minutes to melt the gel and then centrifuged at 14,000 x g for 30 seconds. The spin filter was washed with 500 µl wash solution, centrifuged at 14,000 x g for 30 seconds and again for 1 minute to dry the filter. The DNA was eluted by adding 25 µl of elution solution, flicking the tube and centrifuging at 14,000 x g for 30 seconds.

2.3.7 DNA sequencing

Reactions were done with Sequenase kit (Amersham Pharmacia Biotech). 2-2.5 µg of plasmid DNA (in 25 µl) were denatured by adding 6 µl of 2 M NaOH and heating at 65°C for 10 minutes. The mixture was incubated at room temperature for 10 minutes before adding 42 µl ddH₂O and 9 µl 3 M NaAc (pH 5.2). The DNA was precipitated by adding 2.5 volumes of 100% ethanol at −70°C for 15 minutes, washed with 70% ethanol, allowed to air-dry and then resuspended in 7 µl of ddH₂O. To the DNA 2 µl of T7 Sequenase reaction buffer (200 mM TrisHCl pH 7.5, 100 mM MgCl₂, 250 mM NaCl) was added, along with 1 µl (0.5 pmol) of primer. The mixture was heated to 65°C for 2 minutes to anneal the primer, cooled down slowly to room temperature over 15-30 minutes and put on ice. The ‘Labelling mix’ (7.5 µM 7-deaza-dGTP, 7.5 µM dCTP, 7.5 µM dTTP) was diluted 1 in 5 and 2 µl added to the DNA along with 1 µl 0.1 M DTT, 0.5 µl α-³⁵S dATP and 2 µl of diluted (1 in 8) T7 Sequenase polymerase (13 units/µl in 20 mM KPO₄ pH 7.4, 1 mM DTT, 0.1 mM EDTA, 50% glycerol) and incubated at room temperature for 5 minutes. Tubes with 2.5 µl each of either ‘G’, ‘A’, ‘T’ or ‘C’ termination mix were heated to 37°C, 3.5 µl of the labelling reaction added and incubated at 37°C for 5 minutes. The reactions were stopped by adding 4 µl of ‘stop solution’ (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF).

2.3.8 Polyacrylamide gel electrophoresis of DNA

The sequence reaction samples were heated to 75°C for 3 minutes and then loaded onto 6% denaturing polyacrylamide gels. The gels were run at 60 watts (750-1500
volts) for up to 7 hours in 1 x TBE, dried and exposed to x-ray film (Kodak/Sigma) overnight.

2.4 PCR protocols

Polymerase chain reactions (PCR) were done on the pC89 phagemid with a sense primer sequence designed from the pLac promoter sequence and an antisense primer sequence designed with a BamHI restriction site on its 5' end and the nucleotide sequence for GnRH 3' to it (sequences in Appendix A). A standard PCR contained 2 μg (equivalent to 0.5-1 pmol) of plasmid DNA, 1 x Pfu polymerase buffer, 25 mM dNTPs, 2.5 units of Pfu polymerase and 5 pmol of each primer per reaction. The samples were covered with mineral oil and the PCRs were done in a thermal cycler (Perkin Elmer): 94°C hot-start for 5 minutes; 30 cycles of 94°C for 1 minute, 62°C for 45 seconds and 72°C for 1 minute; a final elongation at 72°C for 5 minutes and then left at 4°C until analysis. The Pfu polymerase was used as it has high fidelity. The PCR products and the pC89 phagemid were digested with the restriction enzymes XbaI and BamHI and ligated in a molar ratio of 1:3, plasmid to insert. JM101 E. coli cells were transformed with the ligation mixture. After identifying the desired GnRH containing plasmids, the sequence of the insert was confirmed by DNA sequencing.

2.5 RNA protocols

2.5.1 DEPC treatment of solutions for RNA handling

Diethylpyrocarbonate (DEPC) to a final concentration of 0.1% (v/v) was added in a fume hood to solutions used for RNA handling. The solutions were left shaking overnight and the excess of DEPC destroyed by autoclaving.
2.5.2 RNA extraction

Tissue samples (heart, liver, seminiferous vesicles and testes) from mice were homogenised by ultrasonication in 3 ml of solution D in a 15 ml tube. 300 μl of 2 M NaAc (pH 4.1) was added along with 3 ml of phenol (water saturated) and mixed. 1.5 ml of chloroform/isoamyl alcohol was subsequently added and mixed vigorously for 10 seconds. The solution was incubated on ice for 15 minutes before being centrifuged for 20 minutes at 3000 x g, 4°C. The aqueous phase was transferred to a new 15 ml tube and an equal volume of isopropanol added, mixed and left at -20°C for 1 hour to precipitate the RNA.

The RNA was collected by centrifugation at 3000 x g for 15-30 minutes at 4°C, washed with 70% ethanol and air-dried. The RNA was then dissolved in 500 μl of solution D and transferred to a 1.5 ml Eppendorf tube and extracted with an equal volume of phenol:chloroform (1:1).

The RNA phase was transferred to a new tube and precipitated with an equal volume of isopropanol for 1 hour at -20°C. The RNA was harvested at 10,000 x g, 4°C for 20 minutes, washed with 70% ethanol and air-dried. The pellet was redissolved in 200 μl DEPC treated water and reprecipitated with 3 volumes of ethanol at -20°C for an hour after addition of 40 μl 3 M NaAc (pH 5.6). The RNA was collected by centrifugation at 10,000 x g, 4°C for 20 minutes, washed with 70% ethanol and air-dried. The pellet was finally resuspended in 200 μl DEPC treated water. The RNA samples were kept as ethanol precipitates at -20°C.

Before the last precipitation a 10 μl aliquot of RNA sample was removed to quantify the amount of extracted RNA, by measuring the O.D. at 260 nm (1 O.D.260 is equivalent to 40 μg/ml of RNA).

2.5.3 RNA agarose gel electrophoresis

Agarose was dissolved in 77 ml of DEPC treated water at concentrations of 1.2-1.5%. 5 ml of 20 x MEA buffer was added and the bottle placed at 62°C for 10 minutes. 18 ml of formaldehyde was added just before the gel was poured in a fume hood.
The required volume of ethanol precipitated RNA (the equivalent to approximately 5μg of RNA) was centrifuged at 10,000 x g for 10 minutes at 4°C to collect the RNA. The pellet was washed with 70% ethanol and air-dried before being resuspended in 5μl DEPC treated water. 10μl of RNA loading dye was added and the samples heated to 80°C for 3 minutes, snap-chilled on ice for 2 minutes, spun down briefly and loaded onto the gel. The gels were run at approximately 80 volts for 1-2 hours in 1 x MEA buffer.

2.5.4 RNA ‘slot-blots’

Forty μg of ethanol precipitated RNA were collected by centrifugation at 10,000 x g, at 4°C for 20 minutes, washed with 70% ethanol and air-dried. The RNA pellets were resuspended in 25μl of DEPC treated water and mixed with 49μl of formamide, 16μl of formaldehyde and 10μl of 10 x MOPS buffer. The RNA samples were heated at 65°C for 5 minutes and placed on ice for 5 minutes to denature any secondary structure. Finally 100μl of ice-cold 20 x SSC was added to the RNA samples. The RNA samples were blotted onto Hybond-N nylon membrane in duplicate 50μl samples using a commercial slot-blotter (BioRad) and the wells washed out afterward with 10 x SSC. The RNA was finally covalently linked to the membrane by UV crosslinking.

2.5.5 Hybridisation of RNA blots with radioactive probe

The RNA blots were pre-hybridised in hybridisation solution at 65°C for at least one hour. The radioactive probe was made using a specific nucleic acid fragment labelled (32P) with Rediprime II Labelling System (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The denatured template was added to the reaction mixture to a final volume of 45μl, the tube flicked and spun down briefly before adding 5 μl of Redivue [32P]dCTP (Amersham Pharmacia Biotech). The reaction was incubated at 37°C for 10 minutes.

The probe was added to the membrane in the hybridisation solution and incubated at 65°C overnight. The membrane was washed in 2 x SSC, 0.1% SDS solution once or
twice for 10 minutes at 65 °C and then exposed to x-ray film for varying amounts of time at −70°C.

The RNA slot-blot membranes were also exposed to a phosphorimager screen and analysed in a Storm phosphorimager (Amersham Pharmacia Biotech) using ImageQuant software (Amersham Pharmacia Biotech).

The membrane was stripped of the radioactive probe with 50 ml of boiling 0.5% SDS solution and allowing it to cool down to room temperature. The membrane was then washed briefly in 2 x SSC before being re-probed.

2.6 Hapten-carrier conjugation

2.6.1 GnRH-TT and GnRH-KLH

GnRH was coupled to either TT or KLH using methods given in protocols from Pierce and Warriner (Perbio). 1 mg of GnRH was reconstituted in approximately 500 μl of EDC conjugation buffer and mixed with 200 μl of 10 mg/ml TT or KLH. 50 μl of 10 mg/ml 1-Ethyl-3-[3-Dimethylaminopropyl]carbodiimide Hydrochloride (EDC, conjugation reagent) was added to the mixture and incubated for 2 hours at room temperature. The solution was centrifuged at 10,000 x g for 2 minutes to remove any precipitated material, before the mixture was gel filtrated through a PD-10 de-salting column (Amersham Pharmacia Biotech), containing Sephadex G-25 M, to separate carrier conjugated GnRH and free carrier from unreacted GnRH. The carrier conjugated GnRH and free carrier were eluted, while the unbound GnRH was retained in the column.

The efficiency of the coupling was examined using a 'dot-blot'. 5 μl aliquots of the conjugation samples at different dilutions were spotted onto Hybond-C (Amersham Pharmacia Biotech) nitrocellulose membrane and air-dried. GnRH and TT were used as positive and negative controls, respectively. After 1 hour at room temperature the membranes were blocked in 5% (w/v) Marvel (skimmed milk powder) in 0.1% (v/v) Tween20/PBS for 30 minutes at 37°C. The membrane was subsequently washed in
0.1 % Tween20/PBS once for 15 minutes and twice for 5 minutes before being probed with the primary antibody: polyclonal rabbit anti-GnRH antibody (Chemicon) at a dilution of 1:10,000 in 5% Marvel/0.1% Tween20/PBS, for 1 hour at room temperature. The excess of antibody was removed by washing as before and then probed with the secondary antibody: anti-rabbit-IgG conjugated with HRP at a dilution of 1:1000 in 5% Marvel/0.1% Tween20/PBS, for 30 minutes at room temperature. The membrane was washed as before and developed with a chemiluminescence reagent, ECL (Amersham Pharmacia Biotech) and exposed to x-ray film for a few seconds.

2.6.2 GnRH-D6-Lys-Hsp70 and hCG-Hsp70

GnRH-D6-Lys was conjugated to Hsp70 using a glutaraldehyde (GDA) coupling method (Avrameas et al., 1978). 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. GDA was added to a final concentration of 0.02% (v/v). The conjugation reaction was left at room temperature, shaking gently for 2 hours. The reaction was then dialysed against PBS in a dialysis cassette (Pierce Warriner (Perbio)) at 4°C overnight. The quality of the conjugation was analysed by SDS gel electrophoresis using a 10% polyacrylamide gel, followed by Western blotting as described above (see section 2.10.2).

Human CG (0.46 mg) was conjugated to Hsp70 (1.0 mg) at a final concentration of 1.0% (v/v) GDA using the same coupling method.

2.7 Phage display protocols

2.7.1 Extraction of phage particles

10 ml of LB medium was inoculated with a fresh colony of *E.coli* JM101 cells containing the phagemid pC89-GnRH and incubated at 37°C, shaking, overnight. The next day 200 ml of LB medium was inoculated (to A<sub>600</sub> = 0.03) with the overnight culture and grown at 37°C, shaking until the culture had reached A<sub>600</sub> = 0.5. The
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culture was then infected with either R408 helper phage (Stratagene) or wild type M13 phage at a multiplicity of infection of 20:1 (phage to cells) and grown until the $A_{600}$ reached 0.9. The expression of the recombinant gene for protein VIII was induced with 1 mM of IPTG and grown further overnight.

The next day bacteria were removed by centrifugation at 10,000 x g for 15 minutes twice before the phages were harvested by adding 13% PEG, 2.5 M NaCl to the supernatant. After 1 hour at 4°C the phages were collected by centrifugation at 10,000 x g for 1 hour. The phage particles were resuspended in 15 ml of T.E. (pH 8.0) and filter-sterilised through a 45 μm filter.

2.7.2 Titration of phage samples

A 5-10 ml culture of JM101 cells was grown at 37°C until the $A_{600}$ reached 0.9. 125 μl of serially diluted phage samples was mixed with 125 μl of JM101, incubated at 37°C for 10 minutes and then put on ice. 3 ml of 2xYT top agar was added, poured immediately over a 2xYT agar plate and the cells grown overnight at 37°C. The next day plaques were visible and the plaque forming units (PFUs) determined.

2.8 Tissue culture

2.8.1 CHO cell transfection

Chinese hamster ovary (CHO) cells were grown in DMMO medium (Gibco BRL), supplemented with L-Glutamine (1% v/v), Penicillin/Streptomycin (1% v/v) and foetal calf serum (FCA, 10% v/v). Cells were maintained in exponential growth by splitting them every 2-3 days. On the day before transfection the cells were seeded onto 10 cm² plates at a density of 2x10⁶ cells/plate. The next day the cells were transfected with 5-20 μg of plasmid DNA per plate, using calcium phosphate precipitation. The DNA was added to 0.25 M CaCl₂ in 1 ml and then to 1 ml of HEPES-PO₄. The DNA solution was then added to the cells after the medium had been removed and left at room temperature for 20 minutes. After 20 minutes 8 ml of complete medium was
added to each plate and incubated overnight at 37°C. The next day the medium was
removed, the cells washed gently with PBS and fresh medium added and left overnight
at 37°C. On the following day the cells were split and diluted 1:10, 1:100 and 1:1000
and medium added along with 1 µg/ml Neomycin (G418, Gibco BRL). The cells were
incubated for a further 7-10 days at 37°C until discrete foci appeared. Individual foci
were picked and expanded to confluency, the supernatant and the cells were harvested
and then tested for expression of rat LH.

2.8.2 CHO cell storage

Confluent cells were trypsinised using 2-5 ml of trypsin solution at 37°C for 5 minutes.
Then the cells were transferred to a 15 ml tube, 10-15 ml PBS added and centrifuged at
5000 x g for 5 minutes. The supernatant was taken off and the cells resuspended in
complete medium, but with 30% FCS, instead of 10%, and 10% (v/v) DMSO added.
The cells were frozen down at approximately 1x10^6 cells/ml in cryotubes and stored at
-80°C or in liquid nitrogen.

2.9 Immunisation protocols

2.9.1 Preparation of the immunogens

For immunisation of rabbits

Ribi adjuvant (Sigma) is a stable oil-in-water emulsion (2% squalene oil-in-water) and
contains monophosphoryl lipid A (MPL), synthetic trehalose dicorynomycolate (TDM)
and cell wall skeleton from mycobacteria (CWS). The conjugates, 2ml, were directly
added to 10 ml of Ribi adjuvant and vortexed vigorously for 2-3 minutes to form an
emulsion.
For immunisation of mice

The Ribi adjuvant system used for immunisation of mice was identical to that described above except it lacked the CWS. The immunogens were mixed with either Ribi as before or Freund’s incomplete adjuvant (FIA, Sigma). The immunogens were added to an equal volume of FIA while vortexing and then vortexed vigorously until a thick emulsion formed. FIA is a water-in-oil emulsion, consisting of paraffin oil and mannide monoleate.

2.9.2 Immunisation schedule of rabbits

New Zealand White (NZW) rabbits were housed at Biological Services UCL under the care of Mr. P. Levy. They were immunised both by intra-muscular (i.m.) and sub-cutaneous (s.c.) injections. The immunisations and bleeds were done by Mr. P. Levy.

Four NZW rabbits were pre-bled and immunised with GnRH coupled to either KLH or TT in Ribi. The pre-bleed and priming were done on the same day (week 0). 250 µl per animal (approximately 50 µg of GnRH) of the mixture was injected at two sites, intra-muscularly and sub-cutaneously. A boost with the same amount of immunogen was done at week 4 (as priming) and the first test bleed was done at week 6. A second boost with the same amount of immunogen was done at week 8 (as priming) and a second test bleed was done at week 10. The rabbits were bled out at week 12.

The blood was coagulated at 37°C for one hour and then left at 4°C overnight. It was then spun down at 5000 x g and the serum collected, aliquoted and stored at -20°C. (Harlow and Lane, 1988)

2.9.3 Passive immunisation of mice

Male BALB/c mice were housed 5 to a cage and kept according to Home Office regulations under the care of Mr. M. Keegan. Immunisations were either done intra-peritoneally (i.p) or sub-cutaneously. At the end of each experiment the mice were sacrificed by a schedule 1 method, exsanguinated by cardiac puncture and dissected. The immunisations and the cardiac punctures were performed by Mr. M. Keegan.
Two sets of passive immunisations on mice were carried out. In the first set fifteen male BALB/c mice were divided into three groups. Group 1 was passively immunised with rabbit serum from a rabbit immunised with GnRH coupled to TT in Ribi adjuvant (Rl). Group 2 was passively immunised with the same amount of pooled rabbit anti-GnRH serum kindly given to us by Professor R.H. Meloen (RH). Group 3 was injected with normal rabbit serum as a negative control for the other two groups (non-specific binding, NS). Each animal was injected intraperitoneally with 200 μl of a 1 in 2 dilution of serum in PBS. The mice received weekly injections from 3.5 weeks until they were 9 weeks of age when they were killed. One test bleed was done at 6.5 weeks.

In the second set of passive immunisations fifteen male BALB/c mice were divided into three groups of five. Group 18 was injected intraperitoneally with 200 μl of a 1 in 2 dilution of rabbit serum Rl in PBS. Group 19 was also passively immunised with Rl serum and additionally actively immunised with oLH (see section 2.8.9). Group 20 was immunised with non-specific rabbit serum (NS, as negative control). No test bleed was taken until the mice were killed at around 9 weeks of age and blood collected by cardiac puncture.

Serum samples were prepared and stored as above.

2.9.4 Active immunisation of mice

Active immunisations of mice were done in three sets of experiments. In the first set of active immunisations 30 3-week-old male BALB/c mice were divided into six groups. Each mouse in group 1 was immunised with 25 μg of GnRH-MAP (Sigma) along with 8 μg of T-helper epitope (kindly given to us by Professor M. Steward, LSHTM) in Ribi adjuvant. The mice in group 2 were immunised with 25 μg per mouse of an irrelevant MAP (Sigma) in Ribi adjuvant. Mice in group 3 were immunised with the GnRH-TT conjugate in Ribi (equivalent to 50 μg of GnRH per mouse). Groups 4 and 5 received 100 μl of phage extract per mouse (1x10^{13} PFU), GnRH displayed on the phage particles for group 4 and wild-type phage particles for group 5. Mice in group 6 received PBS in Ribi as a control. All the immunisations were done i.p. with 200 μl per mouse. The mice were initially immunised at 3-4
weeks of age (week 0) and then boosted at weeks 2 and 5. They were killed and blood collected by cardiac puncture at week 7 (approximately 10 weeks of age). They were dissected and the testes either fixed in Bouin’s solution for histological analysis or flash frozen for RNA analysis.

In the second set of active immunisations 30 male BALB/c mice were again divided into six groups. The first group (group 7) was immunised with GnRH-D6-Lys-Hsp70 conjugate (20 μg per mouse) in incomplete Freund’s adjuvant (IFA). Group 8 was also immunised with the GnRH-D6-Lys-Hsp70 conjugate (20 μg per mouse), but in Ribi. Group 9 was immunised with hCG-Hsp70 conjugate (20 μg per mouse) in Ribi. Group 10 was immunised with GnRH-Th-MAP (kindly given to us by Dr. K.S. Iyer), 25 μg per mouse in Ribi. Group 11 was immunised with GnRH-MAP (Sigma), 25 μg per mouse, in addition to T-helper epitope (kindly given to us by Professor M. Steward), 8 μg per mouse, in Ribi adjuvant. Group 12 was immunised with PBS in Ribi as a negative control. The immunisations were done as before: 200 μl per mouse, i.p. at weeks 0 (3-4 weeks of age), 2 and 5 and the mice were killed at week 7 (10-11 weeks of age). The blood and tissue samples were prepared and stored as above.

The third set of immunisations was done on 35 male BALB/c mice. They were divided into 7 groups. Group 13 was immunised with oLH (10 μg per mouse) in Ribi adjuvant. Group 14 was immunised with the GnRH-D6-Lys-Hsp70 construct (20 μg per mouse) in Ribi. Group 15 was immunised with a combination of GnRH-D6-Lys-Hsp70 construct (20 μg per mouse) and oLH (10 μg per mouse) in Ribi. Group 16 was immunised with GnRH-Th-MAP (from Dr. K.S. Iyer), 25 μg per mouse and group 17 was immunised with a combination of GnRH-Th-MAP (25 μg per mouse) and oLH (10 μg per mouse) in Ribi. Group 19 was immunised with oLH (10 μg per mouse), in Ribi, and also passively immunised with anti-GnRH rabbit serum (R1), as before, but in separate injections. Group 21 received immunisations with PBS in Ribi as a negative control group. The injections were done as before in a final volume of 200 μl per mouse, i.p., on weeks 0 (3-4 weeks old), 2 and 5 and killed at week 7 (10-11 weeks of age). Blood and tissue samples were prepared and stored as before.
2.10 Testosterone assays

Testosterone assays were done with the radioimmunoassay Coat-A-Count Total Testosterone kit according to manufacturer's instructions (Diagnostic Products Corporation). 100 μl of serum were used in each assay. The serum and the controls were added to tubes coated with testosterone-specific antibody, 50 μl in duplicate, and then 1 ml of radioactive $^{125}$I testosterone added to the same tube. After an incubation of 3 hours at 37°C the tubes were emptied and the amount of radioactive testosterone bound to the antibodies on the tubes was determined by counting for 120 seconds in a gamma counter. The testosterone concentration in the serum was calculated from a logit-log representation of the calibration curve.

2.11 Immunoassays

2.11.1 Enzyme-linked immunosorbent assays (ELISAs)

96 well polypropylene ELISA plates (Maxisorp Nunc) were coated with 100 μl per well of 0.2% (v/v) GDA in phosphate buffer (pH 5.0) and left shaking at room temperature for four hours. The plates were washed three times for ten minutes with phosphate buffer (pH 8.0) before 100 μl per well at 10 μg/ml of relevant antigen in phosphate buffer (pH 8.0) were added and incubated overnight. Alternatively 96 well plates were coated directly with 50 μl per well antigen at 1-10 μg/ml in CBB buffer overnight at 4°C. The wells were washed three times with 0.05% (v/v) Tween20/PBS and incubated with primary antibody in 0.05% Tween20/PBS with 3% (w/v) bovine serum albumin (BSA) for one hour at 37°C. The plates were washed as before and incubated with anti-mouse-IgG conjugated to alkaline phosphatase for one hour at 37°C. The ELISA plates were then washed three times with 0.05% Tween20/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 colour change was monitored in an ELISA plate-reader at a wavelength of 405 nm.
2.11.2 Western blots

10-20% SDS-polyacrylamide gels (see appendix B) were run, with a 5% stacking gel on top on a Mini-Protean 3 electrophoresis system (BioRad). They ran at an average current on 25 mAmperes for about 2-3 hours. Then the proteins were transferred onto a Hybond-C nitrocellulose membrane in a transfer tank at 250 mAmperes for 1 hour. The membrane was then blocked in 5% (w/v) Marvel (skimmed milk powder) in 0.1% (v/v) Tween20 in PBS for 1 hour at room temperature and then probed with primary antibody diluted in 5% Marvel/0.1% Tween20/PBS for 1 hour. The membrane was then washed once for 15 minutes and twice for 5 minutes with 0.1% Tween20/PBS and probed with secondary antibody coupled to horseradish peroxidase (HRP), for one hour at room temperature. The immobilised antibodies on the membrane were detected by an ECL western blotting analysis system that reacts with HRP and emits light as chemiluminescence (Amersham Pharmacia Biotech). The membrane was then exposed to x-ray film for a few seconds and the film developed.

The nitrocellulose membranes were stripped of antibody by adding 0.1 M glycine (pH adjusted to 3.4 with HCl). The membranes were left at 37°C for 30 minutes in the stripping solution. They were then washed as before and then re-blocked and re-probed.

2.12 Histological analysis

Testis samples were fixed in Bouin's solution (Sigma) overnight at 4°C. They were then washed in PBS for 30 minutes at 4°C, 1:1 ethanol:PBS for 30 minutes at 4°C, 50% ethanol for 30 minutes at room temperature and 75% ethanol and 100% ethanol both for 30 minutes at room temperature. The samples were then washed twice in toluene for 30 minutes, once in 1:1 toluene:paraffin wax for 30 minutes at 65°C and twice in wax for 30 minutes at 65°C, before being embedded in wax. The samples were left at 4°C overnight and then sectioned in a microtome. The paraffin embedded samples were sectioned and stained first with haemotoxylin stain and then counterstained with eosin. The sections were picked up onto polysine coated microscope slides (BDH) and left to dry overnight at room temperature and baked at 37°C again.
overnight. Drops of haemotoxylin (Sigma) were dropped onto the specimen and washed off with running tap water. Drops of eosin (Sigma) were also dropped onto the specimen and washed off with running tap water. The slides were washed in 50%, 75% and 100% ethanol consecutively for 5 minutes at room temperature before being incubated twice in xylene for 5 minutes and mounted in DPX (Sigma).

2.13 Statistical analysis

Statistical analysis was done on Excel (Microsoft) and Origin (Microcal Software, Inc.) software. 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 normal distribution the non-parametric Wilcoxon non-paired rank test was used.
Chapter 3  Production of Immunogens
3.1 Introduction

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 keyhole limpet haemocyanin (KLH) overcomes this problem and induces antibodies in immunised animals (e.g. Goubau et al., 1989, Ladd et al., 1994, Ladd et al., 1990). 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 the immune response. A prerequisite for the peptide/carrier collaboration is that the peptide be covalently linked to the carrier. Conjugation of peptide antigen to the carrier protein can either be done chemically or by co-expressing the peptide along with the carrier molecule using recombinant DNA technology, e.g. fused with bacteriophage or viral genes. Irrespective of the method chosen it is paramount to conserve the peptide epitope, so that antibodies recognise the native form of the peptide.

Presensitisation with, or repeated usage of, a given carrier can induce ‘carrier suppression’ of the antibody response to the hapten conjugated to it (see section 1.5) (Herzenberg and Tokuhisa, 1980). Hyporesponsiveness to GnRH conjugated to carriers in mice preimmunised with the carriers was found to be carrier-dependent and strain-dependent (Sad et al., 1991). Using fragments of carrier molecules that include important T-cell epitopes but exclude the dominant B cell epitopes of the carrier molecule may eliminate the problem of epitope-specific carrier suppression. Strain-dependency can be overcome by the use of promiscuous T-cell epitopes that are MHC independent. A promiscuous T-cell epitope has, for example, been derived from the fusion protein of the measles virus and has been used as a component of peptide/T-cell epitope constructs (Partidos and Steward, 1990, Partidos et al., 1992).

Another disadvantage to some carrier molecules is their relatively low immunogenicity and the need for potent adjuvants such as FCA to stimulate the immune response non-specifically. Various ‘adjuvant-free’ systems are being developed to circumvent the need for strong and often toxic adjuvants. Carrier proteins such as the heat shock
production of immunogens

Hsp70 have adjuvant properties and may be used efficiently as carriers in an 'adjuvant-free' system. Immunisations with conjugates of Hsp70 and the malarial synthetic peptide (NANP)_{40} (Lussow et al., 1991) and simian immunodeficiency virus (SIV) peptide antigens (Lehner et al., 2000) resulted in efficient antibody responses to the peptides. The specific immunogenicity and adjuvanticity of Hsp70 is discussed in more detail below.

In this chapter the production of GnRH and LH immunogens is discussed, i.e. the production of the GnRH/carrier and hCG/carrier conjugates along with display of GnRH on the surface of filamentous bacteriophage and the expression of rat LH in CHO cells.

3.2 Production of GnRH immunogens

TT and KLH were conjugated to GnRH using a carbodiimide, EDC. Hsp70 was conjugated to GnRH-D6-Lys using GDA. Besides conjugation to carrier molecules, GnRH was also displayed on the surface of filamentous bacteriophage and in a multiple antigen peptide (MAP) system along with a promiscuous T-cell epitope. The MAPs incorporating the GnRH sequence are discussed further in chapter 5.

Hsp70 was also conjugated to hCG using the GDA coupling procedure. This construct was used to immunise male mice, as antibodies against hCG cross-react with LH and in contrast to LH purified hCG is affordably available commercially. Recombinant rat LH was expressed in Chinese hamster ovary (CHO) cells in order to make recombinant rat LH protein to use in immunisations against LH in mice.

3.2.1 Chemical coupling of GnRH to carriers

Conjugation of GnRH to a carrier molecule is limited by the low number of chemically reactive residues in the native GnRH peptide. The main conjugation methods used to couple native GnRH to protein carriers involved diazotization of the GnRH molecule with coupling through the His^2 and Tyr^5 residues (Pique et al., 1978, Koch et al., 1973, Nett et al., 1973) and carbodiimide reactions, which use the His^2, Ser^4 or Tyr^5 residues
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of GnRH to couple it to a carrier (e.g. Fraser and Gunn, 1974). Other conjugation methods include GDA (Barry et al., 1973) or diisocyanate toluene (Barry et al., 1973). Analogues of GnRH have been made to facilitate the conjugation of specific residues of the peptide to a carrier. Substituting glycine for lysine at position 6 of the native peptide, for example, provides the amino group on lysine for GDA coupling, leaving the N- and C-termini unchanged, which are inaccessible due to the natural cyclisation of the N terminus and the amidation of the C terminus. Numerous other analogues, commonly used for conjugation reactions are commercially available.

As expected from the small size of GnRH the residues used for coupling of the peptide to the carrier affects the immunogenicity of the construct. Silversides and colleagues made analogues of GnRH with cysteine residues at positions 1, 6 and 10 (Silversides et al., 1988). They conjugated the analogues to KLH using a heterobifunctional cross-linking reagent and used these conjugates with the adjuvant Havlogen A (an acrylic acid and polyallylsucrose polymer) to immunise mice. They found that the specificity of the antibodies generated was dependent on the site of conjugation. If the carrier was conjugated to the N-terminus the response was against the C-terminus and vice versa. If it was conjugated to position 6 the response was against both termini. However the potency of the immunogen may be dependent on the coupling residue used, as experiments where the N-terminus of GnRH was conjugated to TT evoked a higher GnRH-specific antibody response in male rats, than when TT was conjugated to position 5 or the C-terminus (Ladd et al., 1994).

**TT and KLH**

Both TT and KLH are widely used as carriers for peptide immunogens. TT is the inactivated exotoxin of *Clostridium tetani* (MW 1.4 - 1.5 x 10^5 Da). It is highly immunogenic (e.g. Reece et al., 1993) and is used worldwide as a vaccine against tetanus. KLH (MW 4.5 x 10^5 - 1.3 x 10^7 Da) is highly immunogenic in many species due to its large molecular mass and has around 300-600 lysine residues (6.9% by mass), available for conjugation to peptides or proteins.

TT and KLH were conjugated to GnRH using a heterobifunctional coupling agent (EDC) as described in section 2.6.1. EDC is a carbodiimide that conjugates peptides to carriers via either their N- or C-terminus or at any carboxyl or amine containing side.
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chain. The reaction involves the production of an activated intermediate of the peptide that then reacts with the carrier (see figure 3.1).

After conjugation the reaction samples were gel filtered into 10 mM PBS through a PD-10 (Sephadex G-25) gel filtration column to remove any unbound GnRH or EDC. The absorbance at 280 nm of the column elute was measured and the aliquots containing the conjugates (large MW compounds, i.e. over $1 \times 10^4$ Da) pooled. Since the molecular weight of KLH is too high for it to be resolved on a polyacrylamide gel, aliquots of the samples were dotted onto a nitrocellulose membrane as described in Materials and Methods (section 2.11.2) along with GnRH and TT as controls. The membrane was blocked and probed with GnRH-specific antibodies. The results are shown in figure 3.2. The conjugation samples and controls were dotted in three different concentrations. The GnRH-specific antibody bound to GnRH and also to the conjugated GnRH/TT and GnRH/KLH, but not to TT. This confirmed that GnRH had been successfully conjugated to the two carriers. The conjugates were kept at $-20^\circ$C until used for immunisations.
GnRH:

\[
p\text{Glu His Trp Ser Tyr Gly Leu Arg Pro Gly NH}_2
\]

Conjugation occurs at either the C- or N-terminus or at any carboxyl- or amine-containing side chains.

**Figure 3.1**

EDC mediated peptide-carrier conjugation

A carboxyl group on the protein reacts with EDC to produce an activated protein intermediate. The activated protein can then react with a peptide amine to form a peptide-carrier conjugate. GnRH can be coupled to a carrier molecule by EDC (a carbodiimide) by its His\(^2\), Ser\(^4\), Tyr\(^5\) and Arg\(^8\) residues.
Figure 3.2
Dot-blot of conjugation samples.

GnRH, GnRH/KLH, GnRH/TT and TT were dotted onto a nitrocellulose membrane in the dilutions 10 mg/ml, 25 mg/ml and 50 mg/ml. The membrane was blocked and then probed with GnRH specific antiserum. GnRH acted as a positive control for the anti-GnRH antibody. TT was dotted as a negative control.
Hsp70

Heat shock proteins are widely distributed molecules found in prokaryotic as well as eukaryotic cells. They serve a number of important biological functions, including intracellular chaperoning of proteins by preventing incorrect folding of newly synthesised polypeptides (for a review see Hartl, 1996). They are also involved in regulating immunoglobulin chain assembly, antigen processing and presentation and assembly of MHC molecules (Pierce et al., 1991, DeNagel and Pierce, 1993, Knittler and Haas, 1992). Hsp70 is one of the most highly conserved cellular proteins (Karlin and Brocchieri, 1998, Gupta and Golding, 1993) and yet it is a major immunogen in infections by a variety of pathogens (Shinnick, 1991, Maresca and Kobayashi, 1994). Epitope analysis has shown that Hsp70 proteins have numerous B and T cell epitopes (Behr et al., 1992, Elsaghier et al., 1992, Peake et al., 1993, Quijada et al., 1996, Requena et al., 1993, Zhong and Brunham, 1992).

Mycobacteria and mycobacterial components are frequently used in adjuvants to enhance the immune response to an antigen. BCG (bacille Calmette-Guérin), an attenuated strain of *M. tuberculosis*, has been effectively used to vaccinate against tuberculosis (ten Dam and Pio, 1982) and the tuberculin purified protein derivative (PPD) conjugated to peptides acts as a carrier molecule for immunisations of animals primed with BCG (Lussow et al., 1990). The immunostimulatory effects of BCG priming and immunisation with PPD could partially be ascribed to the Hsp actively produced by mycobacteria (Lussow et al., 1991). Hsp70 from *M. tuberculosis* can evoke a strong T-cell dependent immune response without the need for external adjuvant or priming with BCG when used as a carrier molecule coupled to a peptide antigen (Barrios et al., 1992, Lehner et al., 2000, Lussow et al., 1991, Suzue and Young, 1996). ATP-treated Hsp70 can be either loaded with (Ciupitu et al., 1998), or covalently linked to (Suzue and Young, 1996, Lussow et al., 1991), peptides to elicit a specific anti-peptide immune response. It does this, at least to some extent, by up-regulating the concentrations 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 (Kim et al., 1998, Hedrick and Zlotnik, 1996).
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Recombinant Hsp70 from *M. tuberculosis* (DNAK type) was conjugated to the GnRH analogue GnRH-D6-Lys (with D-Lys substituted for glycine at position 6) using GDA as a homobifunctional reagent. GDA links proteins together via their lysine ε-amino and N-terminal groups as shown in figure 3.3 (Avrameas et al., 1978).

GnRH-D6-Lys is a small peptide with only one possible conjugation site (Lys⁶), as the N-terminus of the peptide is cyclised and therefore not available to react with GDA. The coupling reactions were done with the peptide in molar excess to the carrier (50:1 peptide to carrier). Various different concentrations of GDA (0.002%, 0.02%, 0.1% and 1.0% v/v) were tried, in order to optimise the conditions of the conjugation reactions between GnRH-D6-Lys and Hsp70. The reactions were separated on 10% non-reducing SDS polyacrylamide gels (see figure 3.4). DTT in the sample buffer was found to disrupt the bond between the peptide and carrier. The proteins were transferred onto a nitrocellulose membrane (Hybond C) and then probed with anti-GnRH and anti-Hsp70 antibodies consecutively. Reactions with a final GDA concentration of 0.02% (v/v) showed the highest intensity of staining with the anti-GnRH antiserum and this concentration was used in the coupling reactions to conjugate GnRH-D6-Lys and Hsp70 for immunisations.

After conjugation at room temperature for two hours the samples were dialysed overnight against PBS and kept at −20°C until they were to be used in immunisations. Just prior to immunisation, aliquots of the conjugation samples (before and after dialysis) were examined by Western blot analysis using both anti-GnRH and then anti-Hsp70 antisera (figure 3.5). When the membrane was probed with anti-GnRH antibodies there was a clear high molecular weight band in the conjugation samples, that corresponded to the size of Hsp70.
The GnRH analogue GnRH-D6-Lys has D-Lys at position 6 instead of glycine. Hsp70 is approximately 6% Lys (w/w). GDA couples GnRH-D6-Lys to Hsp70 via the ε-amino groups of the lysine residues present in the peptide and the carrier.
Production of Immunogens

Figure 3.4
GnRH-D6-Lys/Hsp70 conjugations – Optimisation of the coupling reactions

Conjugation of GnRH-D6-Lys and Hsp70 was done at different concentrations of GDA (0.002%, 0.02%, 0.1% and 1.0%). The reactions were run on 10% SDS gels (w/o DTT), 2 μg of conjugate loaded per lane. The proteins were transferred onto a nitrocellulose membrane and the membrane probed with a polyclonal GnRH-specific antiserum (A) and polyclonal Hsp70-specific antiserum (B).
Before immunisations the conjugation reactions were run on 10% SDS gels (w/o DTT) and the proteins transferred onto a nitrocellulose membrane. The membrane was probed with anti-GnRH antibodies (A), stripped and re-probed with anti-Hsp70 antibodies (B).

The conjugation samples show a band corresponding in size to Hsp70 (approximately 70 kDa) both before and after dialysis.
3.2.2 Phage display of GnRH

Foreign peptides or proteins can be displayed on the surface of filamentous bacteriophage and act as ligands for various biological systems, including antigen-antibody interaction. The DNA sequences for the foreign peptides can be inserted into the 5' end of one of the bacteriophages' coat protein genes, e.g. protein III or protein VIII. Random DNA sequences can be used to create libraries of peptides that can be screened for effector activities (Cwirla et al., 1990, Scott and Smith, 1990).

Filamentous bacteriophages (fd, fl and M13) are non-lytic, i.e. do not lyse their host cells during phage production. They have five coat proteins, four minor and one major. The minor coat proteins are encoded for by genes III, VI, VII and IX. Protein VII (pVII) and pIX are at the end of the phage where phage assembly is initiated, pIII and pVI where it ends (reviewed in Rasched and Oberer, 1986). Protein III is important in initiating infection and attaching the phage particle to the F pilus of the target E.coli bacteria. The minor coat proteins are present in five copies each, whereas around 2700 copies of the major coat protein pVIII form a tubular sheath around the phage DNA.

Display of foreign peptides was initially done by incorporating the peptide DNA in the gene for pIII, near its N-terminus. This method is effective in selection of ligands from large libraries of peptides (Devlin et al., 1990, Cwirla et al., 1990, Scott and Smith, 1990). Incorporating the peptide sequence into the gene for the major coat protein pVIII has the advantage that the pVIII is present in much higher numbers on the surface of the phage and would potentially be able to display peptides in higher numbers and density.

Protein VIII is 50 aa long and is mainly α-helical. It can be divided into three main domains on the basis of amino acid composition. The amino terminus is predominantly acidic amino acids while the carboxyl terminus, which interacts with the viral DNA, is characterised by four lysine residues and is positively charged. The middle region (aa 21-39) is mainly hydrophobic. The protein is synthesised with an N-terminal extension of 23 aa that ensures that the protein is transported into the
periplasmic space where it is cleaved off by the enzyme leader peptidase (Hunter et al., 1987).

As long as the peptide sequence inserted into gene VIII does not interfere with translocation across the cell membrane or with phage particle assembly, it can be displayed on the surface of the phage particle. There is however a limit to the length of the displayed peptide. So far 5-8 aa long peptide inserts have been successfully displayed (Malik et al., 1998, Greenwood et al., 1991). To incorporate longer peptides it is necessary to display recombinant pVIII interspersed with wild type (wt) pVIII protein on a phage hybrid (see figure 3.6). In this manner up to 12 aa long peptides can be displayed on the phage surface (Rowitch et al., 1988, Greenwood et al., 1991).

![Phage display of foreign peptides](Image)

**Figure 3.6**
Phage display of foreign peptides

a) A schematic picture of a wild type filamentous bacteriophage.

b) A bacteriophage with only recombinant pVIII on its surface.

c) A hybrid bacteriophage with recombinant pVIII interspersed with wt pVIII.

The bacteriophage particles are themselves highly immunogenic and therefore act as carriers for the peptides displayed on their surfaces, such as peptides representing antigenic determinants of the malarial circumsporozoite protein (de la Cruz et al., 1988, Willis et al., 1993). Phage constructs have been found to be strongly immunogenic in various mouse strains, even without the addition of adjuvants (Willis et al., 1993). The immune response is T-cell dependent, as immunisation of nude (nu/nu) BALB/c mice resulted in a much weaker response, with mostly IgM
antibodies, whereas in a heterozygous strain (nu+/-) the antibodies were both IgM and IgG as expected in a T-cell dependent immune response (Willis et al., 1993).

Cloning of GnRH into pC89

The sequence for GnRH was cloned into a plasmid (pC89) kindly given to us by Professor J. Engberg (Department of Biological Sciences, The Royal Danish School of Pharmacy). The plasmid contained the phage fl gene for pVIII under the control of the pLac promoter, with a random insert between codons 3 and 4 of gene VIII. The phagemid vector system was originally developed with the gene for pVIII fused to the gene encoding the α-peptide of β-galactosidase via an amber codon (Felici et al., 1991).

Since pC89 already contained a random oligonucleotide insert in the EcoRI/BamHI site in gene VIII the GnRH nucleotide sequence was introduced into pC89 first by PCR of the pC89 plasmid producing an oligonucleotide incorporating a BamHI restriction site, the sequence for GnRH and the remaining sequence for the amino end of pVIII. The PCR was done with a forward primer recognising the pLac sequence and a reverse primer including a BamHI site and the sequence for GnRH, as described in section 2.4. The PCR product was run on an agarose gel (see figure 3.7). The 300 bp long PCR product was digested with XbaI and BamHI. The phagemid pC89 was also digested with XbaI and BamHI and ligated with the PCR fragment (figure 3.7). JM101 E. coli cells were transformed with the ligation mix. To test whether the PCR product had been ligated into the phagemid another PCR was done on the extracted phagemids from the newly transformed cells. The PCR reactions were run on an agarose gel and the 300 bp PCR product identified in 10 out of 24 clones (figure 3.7 (C)). One clone (no. 5) was sequenced over the GnRH insert (figure 3.8)
Production of Immunogens

Figure 3.7
Cloning of the oligonucleotide sequence for GnRH into the pC89 phagemid

A) PCR was done using the pC89 (with random nucleotide insert) as template. The sense PCR primer recognises part of the pLac nucleotide sequence. The antisense PCR primer includes the sequence for GnRH and for a BamHI restriction site.

B) The PCR product was run on a 1.5% agarose gel.

C) After digestion of the PCR product and pC89 with XbaI and BamHI the PCR fragment and the pC89 vector were ligated. Verification of the ligation was done by doing a second set of PCRs using the same primers. The products were run on a 1.5% agarose gel. Clones 1-8 and 12-13 are positive.

D) The amino acid sequence of the amino terminus of the recombinant GnRH/pVIII protein expressed on the surface of the phage particles.
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Figure 3.8
Sequencing of GnRH/pC89 clone

The GnRH/pC89 clone was sequenced using the T7 primer. The sequence is shown in the 3' to 5' orientation read from top to bottom of the gel.
Extraction of phage particles for immunisations

Exponentially growing transformed cells were grown overnight in LB medium and then superinfected with wt phage. The pLac promoter is inducible by addition of IPTG to the culture, and along with superinfection with wt phage, should make it possible to vary the ratio of wt to recombinant pVIII on the phage’s surface. Felici and co-workers found that in the bacterium/phagemid system described the addition of IPTG only raised pVIII expression by a factor of 2 (Felici et al., 1991). After growing the cells to the desired density the phage particles were purified from the culture supernatant as described in section 2.7.1, the plaque forming units (pfu) determined and the extracts kept at 4°C.

The extracted phage particles were run on a 20% SDS gel and silver stained (figure 3.9). The major coat protein pVIII is visible as a band of approximately 7000 Da in the wt phage extract, the induced and the uninduced phage extracts. There is no additional band representing a recombinant pVIII protein.

Since no significant amount of GnRH-pVIII was detectable using SDS gel electrophoresis, the presence of recombinant GnRH-pVIII on the phage particles was analysed using an ELISA. The phage particles were used to coat ELISA plates and three different GnRH-specific polyclonal antibodies used to bind to the extract: a commercial anti-GnRH polyclonal antiserum (RC), R9 polyclonal antiserum and RH polyclonal antiserum, all raised in rabbit. The results of the ELISAs are shown in figure 3.10. The plate coated with GnRH on its own (a control for the antisera, graph A) shows that the RH antiserum shows less binding to GnRH than the other two antisera. Non-specific rabbit serum and an hCG-specific antiserum were used as a negative controls, but unfortunately the hCG-specific serum seemed to bind non-specifically to the phage extracts (graphs B, C and D). M13 (bacteriophage)-specific antiserum was used as a positive control for the ELISAs on the phage extracts. The M13 antibody binds to a similar extent to all three phage extract samples (graphs B, C and D). In graphs B and C the ELISA plates have been coated with GnRH/pC89 phage extract, induced with IPTG and not induced, respectively.
Figure 3.9
Silver staining of phage extract run on a 20% SDS polyacrylamide gel

Equivalent titres (pfu) of phage extract were run in each lane (lane 1: RM, lane 2: wt phage, lane 3: induced, lane 4: uninduced phage particles). A separate band with recombinant pVIII is undetectable. This is because the recombinant pVIII is not incorporated into the phage particles at very high concentrations.
Figure 3.10

Verification of expression of GnRH on the surface of phage particles by ELISAs

The plates were coated with
A) GnRH (10 μg/ml)
B) induced phage particles displaying GnRH/pVIII and wt pVIII (approx. $10^{10}$ pfu/ml)
C) not induced phage particles displaying GnRH/pVIII and wt pVIII (approx. $10^{10}$ pfu/ml)
D) wt phage particles (approx. $10^{10}$ pfu/ml)

The antibodies used were three different GnRH-specific polyclonal antisera, one normal rabbit serum, a polyclonal hCG-specific antiserum (as a negative control) and a polyclonal M13 (bacteriophage) antiserum.
In both of these ELISAs there is considerable binding of all three GnRH-specific antisera compared to the non-specific rabbit serum. On the plate coated with wt phage only the M13 antibody and the hCG antibody bound to the wt phage on the plate.

In addition the phage samples were compared on dot-bLOTS with the induced GnRH-phage display particles and wt phage particles, probed with a GnRH-specific antiserum (RC, figure 3.11). The induced GnRH-phage particles show more intense staining, although the antibodies also bind non-specifically to the wt phage extract.
GnRH, induced phage particles and wt phage particles were dotted in three dilutions onto a nitrocellulose membrane (GnRH: 50, 25, 10 μg/ml; phage particles: neat, 1:5, 1:10 dilutions). The membrane was blocked and probed with anti-GnRH antiserum (RC) at a dilution 1:1000 in PBS/0.1% Tween/1.5% BSA.
3.3 Conjugation of hCG to Hsp70

Recombinant Hsp70 (DNAK type) was conjugated to hCG using GDA. The conjugation reactions were done with a molecular ratio of Hsp70 to hCG of 1:2. Reactions using different GDA concentrations (0.01%, 0.1% and 1.0%) to optimise the conjugation reactions were done. The efficacy of the conjugation reactions was tested by Western blot analysis and by capture ELISA. The different reactions were run on 10% SDS polyacrylamide gels, the proteins transferred onto nitrocellulose membranes and then probed with polyclonal anti-hCG serum (figure 3.12). The reactions were also captured on ELISA plates coated with a hCG-specific monoclonal antibody (OT3A) and probed with polyclonal anti-Hsp70 (figure 3.13). On the membrane probed with anti-hCG antibody there were high molecular weight bands only in the lane with hCG/Hsp70 conjugated at a concentration of 1.0% GDA. The capture ELISA also shows that the Hsp70 antibody binds in highest density to the hCG/Hsp70 complex conjugated at 1.0% (v/v) GDA. The reactions were done at room temperature and then dialysed overnight against PBS.

The conjugation reactions to be used for immunisations were run on SDS gels before and after dialysis just prior to the immunisations and probed with both anti-hCG and then anti-Hsp70 antisera (figure 3.14). On the membrane probed with anti-hCG antibodies there was a definite band corresponding to the size of Hsp70 in the hCG/Hsp70 conjugation samples, both before and after dialysis.
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220.0 KDa
97.4 KDa
66.0 KDa
46.0 KDa

Membrane probed with anti-hCG polyclonal antibody

Membrane probed with anti-Hsp70 polyclonal antibody

A

B

hCG/Hsp70

hCG/Hsp70

220.0 KDa
97.4 KDa
66.0 KDa

Hsp70 main band (approx. 70 KDa)

hCG monomer

Figure 3.12
hCG/Hsp70 conjugations – Optimisation of the coupling reactions

Coupling reactions between hCG and Hsp70 were done at different concentrations of GDA. Reactions with 0.1% and 1.0% GDA were run on 10% SDS polyacrylamide gels (w/o DTT), the proteins transferred to a nitrocellulose membrane and the membrane probed first with antiserum against hCG (A) and then anti-Hsp70 antibody (B). The conjugate done with 1.0% GDA shows high molecular weight staining, evidence of conjugation of hCG with Hsp70.
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Figure 3.13
Capture ELISA to detect hCG/Hsp70 conjugates

The ELISA plates were coated with a monoclonal antibody against hCG (OT3A) that captured successfully conjugated hCG/Hsp70. The hCG/Hsp70 was then recognised by an anti-Hsp70 polyclonal antibody raised in macaque. The macaque antibody was recognised by anti-human antibody (raised in goat) and finally the goat anti-human antibody was recognised by an alkaline phosphatase conjugated anti-goat antibody (shown schematically in (A)).

After addition of alkaline phosphatase substrate the absorbance at 405 nm was measured in an ELISA plate reader and represented in a graph showing the dilutions (5 μg/ml, 1 μg/ml, 0.2 μg/ml and 0.04 μg/ml) of the conjugation reactions on the X-axis (B). Hsp70 and hCG were used as controls for the ELISA.
Figure 3.14
hCG/Hsp70 conjugations

Conjugation samples along with hCG and Hsp70 as controls were run on a 10% SDS gel (w/o DTT) just prior to immunisations. The proteins were transferred onto a nitrocellulose membrane, blocked and then probed first with anti-hCG polyclonal antibodies (A) and then with anti-Hsp70 polyclonal antibodies (B). The conjugation samples (both before and after dialysis) show staining with the anti-hCG antiserum of a band corresponding in size to the approximate size of Hsp70. There is also some higher molecular weight staining pointing to the existence of high molecular weight conjugates.
3.4 Expression of rat LH in CHO cells

Recombinant rat LH has been successfully cloned into pKCRE, with both α- and β-subunits under the control of the SV40 promoter and expressed in Chinese hamster ovary (CHO) cells (Hakola et al., 1997a). N-linked oligosaccharides are necessary for the bioactivity of the glycoproteins. Glycoproteins, other than LH (FSH and hCG) produced in CHO cells have very similar oligosaccharide moieties compared with the pituitary proteins (Takeuchi et al., 1989). LH on the other hand has mainly sulphated, not sialylated, carbohydrates and as CHO cells lack N-acetylgalactosamine transferase and sulphotransferase (Smith et al., 1990), the terminal carbohydrates of recombinant rat LH are sialylated. The rec rat LH was however found to have higher in vitro potency than pituitary rat LH (Hakola et al., 1997a).

In order to produce rat recLH to use in immunisations of mice against LH, CHO cells were transfected with the α/LHβ/pKCRE plasmid (kindly given to us by Dr. W. Schoonen, N.V. Organon, Oss, The Netherlands) using the procedure described in section 2.8.1. Before transfection the plasmid was extracted and digested with various restriction enzymes to verify the α/LHβ insert in the pKCRE vector. The results of the restriction analysis are shown in figure 3.15 (A). The gel showed bands of the predicted sizes. Additionally a PCR reaction was done using the plasmid as a template and a sense primer recognising the α-subunit of LH and an antisense primer recognising the β-subunit of LH. A PCR product corresponding in size to the α- and β-subunits of LH in pKCRE was detected on a 1.5% agarose gel (figure 3.15 (B)). Large-scale preparations of the plasmid were made for the CHO cell transfection. Transfection and selection of αLHβ/pKCRE expressing clones was done as described in section 2.8.1.

The cell supernatant was harvested and the expression of recombinant rat LH and secretion into the supernatant verified by running the supernatant on 15% SDS gels. The proteins were transferred onto a nitrocellulose membrane and then probed with rat LH-specific antibody (figure 3.16). No specific recombinant rat LH band was detected on the gels.
Production of Immunogens

**Figure 3.15**
Cloning of αLHβ/pKCRE

A) Restriction enzyme analysis of the αLHβ/pKCRE plasmid

The plasmid was digested in nine digestions and the enzymes used are listed (1-9 in list) along with the predicted sizes of fragments produced by these digests.

<table>
<thead>
<tr>
<th>Restriction enzyme(s)</th>
<th>Predicted fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EcoRI</td>
<td>5.0, 2.3</td>
</tr>
<tr>
<td>2. Sail</td>
<td>7.4</td>
</tr>
<tr>
<td>3. BamHI</td>
<td>4.7, 2.0, 0.4, 0.4</td>
</tr>
<tr>
<td>4. Pvul</td>
<td>7.4</td>
</tr>
<tr>
<td>5. EcoRI + Sail</td>
<td>2.7, 2.4, 2.3</td>
</tr>
<tr>
<td>6. EcoRI + Pvul</td>
<td>4.5, 2.3, 0.6</td>
</tr>
<tr>
<td>7. EcoRI + BamHI</td>
<td>4.6, 1.9, 0.4, 0.4, 0.08, 0.08</td>
</tr>
<tr>
<td>8. Sail + BamHI</td>
<td>2.8, 2.0, 1.9, 0.4, 0.4</td>
</tr>
<tr>
<td>9. Sail + PvuI</td>
<td>5.3, 2.1</td>
</tr>
</tbody>
</table>

B) PCR verification of the αLHβ/pKCRE construct

- 0.6 bp PCR fragment
Figure 3.16
Supernatant off CHO cells transfected with αLHβ/pKCRE plasmid run on a 15 % SDS gel

Stable cell lines, transfected with αLHβ/pKCRE and selected by Neomycin, were grown and the supernatant harvested (lanes 1-7). Supernatant off non-transfected cells (lane 8) was run as a control. 1.0 ml of supernatant was concentrated on Stratclean resin (Stratagene) to concentrate the proteins from the supernatant to a volume of 20μl that was loaded onto the gel. The proteins were transferred onto a nitrocellulose membrane, the membrane was blocked and then probed with rat LH-specific polyclonal antiserum.
3.5 Discussion

Constructs of GnRH and hCG with carriers were made for immunisations of male mice. GnRH was coupled chemically to the carrier molecules TT, KLH and Hsp70 and expressed on the surface of filamentous phage particles using recombinant DNA technology. Human CG was coupled chemically to the carrier Hsp70. TT and KLH were successfully coupled to the native form of the GnRH peptide using a carbodiimide conjugating reagent, EDC. A dot-blot with the conjugated peptide/cARRIER complexes and GnRH and TT as controls was probed with anti-GnRH antibody. The antibodies recognised both conjugates, GnRH/TT and GnRH/KLH but did not bind to TT on its own. Unbound GnRH had been removed from the conjugation samples by gel filtration so the GnRH-specific antibodies bound only peptide/cARRIER complexes.

Silversides and colleagues found that antibody responses to synthetic GnRH analogues conjugated to carriers at position 6 or 10 of the peptide were higher than those conjugated at position 1, i.e. antibodies raised against the C-terminus or both termini (Silversides et al., 1988). Hsp70 was conjugated to the GnRH analogue GnRH-D6-Lys at position 6 using GDA. Antibody responses against this peptide/cARRIER complex should therefore be mounted against both N- and C-termini of GnRH. The optimal concentration of GDA for GnRH-D6-Lys/Hsp70 coupling was found to be 0.02%. Interestingly, at higher concentrations of GDA GnRH was bound to Hsp70 to such an extent that all B cell epitopes on Hsp70 became inaccessible to the antiserum and Hsp70 was not detected in the conjugates by Western blot analysis. Other workers have used both higher and lower GDA concentrations (Lussow et al., 1991, Avrameas et al., 1978) and as each peptide/cARRIER conjugation system requires different conditions it is necessary to optimise the reactions for each case.

Synthesis of peptides and chemical conjugation of peptides to carriers can be expensive, variable and time consuming. Foreign peptides can be inserted into the pVIII coat protein of the filamentous bacteriophage using recombinant DNA technology. This expression-exposition system has the advantages of being highly
immunogenic, even without added adjuvant (di Marzo Veronese et al., 1994, Greenwood et al., 1991) and easily purified, as the phages are non-lysogenic and the phage particles are extruded into the host cell culture supernatant. In the supernatant there are also immunostimulatory factors from the host cells such as *E.coli* lipopolysaccarides (LPS) and *E.coli* RNA.

The nucleotide sequence for GnRH was successfully cloned into the pC89 phagemid, at the N-terminus end of the gene encoding the pVIII coat protein. The pVIII gene in pC89 is under the control of the pLac promoter and the expression of the protein is therefore inducible by addition of IPTG. Superinfection by wt phage and modulation of pLac activity should facilitate the construction of chimeric phages with different densities of recombinant pVIII to wt pVIII on their surfaces. Induction with IPTG did not result in increased incorporation of GnRH/pVIII fusion protein into phage particles and there was evidence for recombinant pVIII and wt pVIII being produced even without IPTG induction. These results were consistent with Felici and co-workers, who found that addition of IPTG to this particular bacterium/phagemid system only raised the pVIII expression by a factor of 2 (Felici et al., 1991).

The phage particles were grown in the host cells and extracted from the cell supernatant. The extracts were run on SDS-polyacrylamide gels, that were then silver stained. The recombinant pVIII should be detectable in the hybrid phage particles as a separate band in addition to the pVIII band (e.g. Greenwood et al., 1991). The GnRH-phage particles however only had one band corresponding to the major phage protein pVIII. This is consistent with some other investigators who found that due to unusual electrophoretic properties of pVIII the separation of wt and recombinant pVIII was very difficult in a number of gels systems (Felici et al., 1991). The hybrid phage particles could also have too low a proportion of recombinant GnRH/pVIII to wt pVIII to be detected by SDS electrophoresis. Immunostaining showed that an anti-GnRH antiserum bound to the GnRH/phage particles on a nitrocellulose membrane and the expression of GnRH on the surface of the phages was verified by ELISA.

Three GnRH-specific polyclonal antisera all recognised the GnRH/phage extracts on the ELISA plates and there was no apparent cross-reaction with the wt phage extract. The bacteriophage (M13)-specific antiserum recognised all the phage extracts and did not cross-react with GnRH. Non-specific rabbit serum was used as a negative control.
Production of Immunogens

for the GnRH and M13 antisera (all raised in rabbit) and did not bind to any of the antigens coating the ELISA plates. Human CG-specific rabbit antiserum was also used as a negative control, but for unknown reasons it bound to all the phage extracts (recombinant and wt).

The recognition by the anti-GnRH sera of the recombinant phage extracts and its non-reactivity to the wt phage extracts verifies the display of GnRH peptide on the surface of the phage particles and that the GnRH epitopes are conserved and available for interaction with antisera in the construct. The recombinant pVIII coat proteins, with the GnRH peptide insert, were translocated, processed and incorporated efficiently into a bacteriophage capsid interspersed with wt pVIII.

Human CG was conjugated to Hsp70 using GDA. As with the GnRH/Hsp70 conjugation reactions a few different concentrations of GDA were used to optimise the reaction. The hCG/Hsp70 complexes were run on SDS-polyacrylamide gels and probed with specific anti-hCG and anti-Hsp70 antibodies and also captured on ELISA plates and probed with anti-Hsp70 antiserum. The gels showed that the anti-hCG antiserum bound to high molecular weight bands (>7 x 10^4 Da) when the reactions were done with 1.0% (v/v) GDA. The capture ELISA verified this finding as the only hCG/Hsp70 complex that was recognised by anti-Hsp70 antibody was that conjugated with 1.0% GDA. The optimal concentration of GDA for the hCG/Hsp70 conjugation reactions is considerably higher than that needed for GnRH/Hsp70 conjugation (0.02% GDA (v/v)).

Rat recombinant LH was expressed in CHO cells. A restriction enzyme analysis of the αLHβ/pKCRE plasmid verified the cloning of the α- and β-subunits of rat LH into the pKCRE plasmid under the control of the SV40 promoter. CHO cells were transfected transiently with the construct and grown as described in section 2.8. The supernatant from the transfected cells was run on a SDS polyacrylamide gel and probed with anti-rat LH antiserum. Unfortunately no recombinant rat LH was detected in the cell supernatant. This could be due to low expression of rat LH in the CHO cells. In previous experiments expressing recombinant rat LH and rat FSH in CHO cells the level of expression of recombinant protein was extremely low (approximately 1 mg/l of cell supernatant) and large amounts of cell supernatant have to be used to extract
detectable amounts of protein (Hakola et al., 1997a, Hakola et al., 1997b). Large scale production of rat LH was impossible as the facility to do so was unavailable.

The immunogens were stored at -20°C (conjugates of GnRH/TT, GnRH/KLH, GnRH/Hsp70 and hCG/Hsp70) and at 4°C (phage extracts) until they were used for immunisations.
Chapter 4  Raising Antibodies and Passive Immunisations
4.1 Introduction

Passive immunisation of male animals with antisera against GnRH has been shown to be effective in inhibiting GnRH action and reducing serum LH and testosterone levels (e.g. Lincoln and Fraser, 1979, Hauger et al., 1977, Bercu et al., 1977). Passive immunisation has the advantage that potentially neutralising antibodies are available immediately, without the lag time of the induction of an active immune response. Passive immunisation is however impractical for routine use, as large amounts of antisera are needed in numerous injections to sustain the level of antibody in the immunised animal.

The effects of passive immunisation with anti-GnRH antibodies on the reproductive system of the host are indicative of complete neutralisation of GnRH in the animals. GnRH is necessary for the normal development of the hypothalamic-testicular axis and neutralisation of GnRH by passive immunisation with GnRH-specific antiserum in pre-pubertal animals resulted in decreased testicular weight and impaired fertility (Bercu et al., 1977). Vogel and co-workers also found that GnRH was required for normal pre-pubertal sexual development and that fertility was impaired in passively immunised animals even though mature spermatozoa were detectable both in the seminiferous tubules and the epididymis (Vogel et al., 1983).

At puberty serum testosterone levels in male animals rise drastically, regulated to an extent by the increase in serum LH concentrations. Before making an experimental protocol for passive immunisations of male mice serum testosterone levels were determined as a function of time in a cohort of male mice. Testosterone was measured at two-week intervals (2, 4, 6, 8, 10 and 12 weeks).

Antiserum specific for GnRH was produced in rabbits by immunising them with GnRH conjugated to two different carriers, TT and KLH. To determine whether the onset of puberty, measured as testosterone level and testes weight, could be affected pre-pubertal male BALB/c mice were passively immunised with the GnRH-specific rabbit sera.
4.2 Results

4.2.1 Raising antibodies against GnRH in rabbit

Four New Zealand White (NZW) rabbits were immunised with GnRH peptide conjugated to carriers together with Ribi adjuvant. Two animals (R1 and R2) were immunised with GnRH conjugated to TT and two (R3 and R4) with GnRH conjugated to KLH following the immunisation schedule described in section 2.9.4. The rabbits were bled and the serum extracted. Antibody levels were measured by direct ELISA. The ELISA plates were coated with GnRH, the rabbit serum recognised and bound to the peptide and anti-rabbit antibody, conjugated with alkaline phosphatase bound to the rabbit antibodies. The absorbance at 405 nm was plotted against the serial dilutions of the serum (figure 4.1 A). The antibody endpoint titres were taken to be the serum dilution that gave the same O.D. value as the mean of the pre-bleed serum plus three standard deviations (S.D.) from all four rabbits. All the rabbits showed antibody production, although one GnRH-KLH immunised rabbit had only marginal levels of GnRH-specific antibodies. The two immunised with GnRH-TT responded with considerably higher endpoint antibody titres of 3000 than the two immunised with GnRH-KLH with endpoint titres of 150 and 50 respectively (see figure 4.1 B).

Professor R. H. Meloen (Institute for Animal Science and Health, Lelystad) supplied us with serum from rabbits immunised with a multivalent construct of GnRH conjugated to KLH (RH). The antiserum was used to passively immunise mice, along with the rabbit serum raised against GnRH-TT (R1). The two antisera were compared in an ELISA (figure 4.2). The R1 serum showed considerably higher binding to GnRH on the ELISA plate and had an approximately 8 times higher antibody titre than RH.
Figure 4.1
Active immunisation of rabbits with GnRH conjugates

Two NZW rabbits, R1 and R2, were immunised with GnRH-TT conjugate. Two, R3 and R4, were immunised with GnRH-KLH conjugate. Antibody titres were measured by ELISA. Normal rabbit serum (NS) was used as a control for non-specific binding.

A) GnRH specific antibody titration curves determined by ELISA on plates coated with GnRH and anti-rabbit IgG antibody conjugated to alkaline phosphatase. Absorbance (O.D. 405 nm) is plotted against serum dilutions.
B) Endpoint titres were determined as the serum dilution with an O.D. value equal to the mean of the control samples (n=5) plus three standard deviation from the mean.
Figure 4.2
Comparison of the GnRH specific antibody titration curves for the rabbit sera

R1 was raised in rabbit after immunisation with a conjugate of GnRH and TT. RH (a gift from Professor R.H. Meloen) was raised in rabbit after immunisation with a multivalent GnRH construct conjugated to KLH. NS is normal rabbit serum. The rabbit sera were applied to GnRH-coated plates and subsequently probed with alkaline phosphatase-conjugated anti-rabbit polyclonal antibodies.
4.2.2 Passive immunisations of mice with anti-GnRH rabbit sera

At the time of puberty plasma testosterone levels in male mammals rises dramatically (e.g. Selmanoff et al., 1977). Before deciding on the experimental procedure for passive immunisations plasma testosterone levels at various time-points were measured in a cohort of male mice (figure 4.3). There is a marked increase in testosterone levels from approximately 5 to 50 nmol/l around the age of 6-8 weeks, although the levels vary widely between individual animals. The rise coincides with the age that the mice reach puberty. Passive immunisations were started when the mice were around 4 weeks old, well before the onset of puberty. The commencement of gonadotrophin pulses at the time of puberty is dependent on the onset of GnRH pulsatility (Wildt et al., 1980, Ross et al., 1983). Total neutralisation of GnRH would halt the development of the gonads and accessory organs and inhibit production of testosterone and spermatogenesis.

Three groups of five pre-pubertal male BALB/c mice were passively immunised with rabbit serum. Group 1 was injected with anti-GnRH serum R1. Group 2 was injected with anti-GnRH serum RH. The mice received weekly injections of antiserum for six weeks. Unfortunately one of the mice in group 2 died for unknown reasons 2-3 weeks into the experiment. Group 3 received injections of non-specific rabbit serum (NS).

A test bleed was taken at 6.5 weeks of age (3 weeks after initial injection), by tail bleeds. The mice were bled out at 9.5-10 weeks of age (6 weeks after initial injection). Serum was extracted from the whole blood as before and the rabbit antibody levels in the mouse sera from the final bleeds were measured by ELISA (see figure 4.4). Testosterone levels in the sera were measured by RIA (see figure 4.5). The testes were weighed and prepared for histological analysis.

The ELISAs showed that the mice in group 1 had higher rabbit anti-GnRH antibody levels than the mice in groups 2 and 3 (figure 4.4), which is consistent with the fact that the R1 serum contained considerably higher titres of GnRH-specific IgG than the RH serum (figure 4.2).
Raising Antibodies and Passive Immunisations

Figure 4.3
Testosterone levels in mouse sera at various ages

Test bleeds were taken every 2 weeks from three male Balb/c mice and testosterone levels measured by RIA. Testosterone levels rise rapidly around 6-8 weeks, which coincides with the age that mice reach puberty.
Figure 4.4
Residual GnRH-specific IgG antibodies in male BALB/c mice passively immunised with anti-GnRH specific rabbit sera

A) ELISA plates were coated with GnRH, probed with mouse sera and re-probed with alkaline phosphatase conjugated anti-rabbit polyclonal antibody. Mice in group 1 (P1), who received R1 rabbit serum, were numbered P1a-e and in group 2 (P2), who received RH rabbit serum, P2a-d.

B) The endpoint antibody titres were taken to be the serum dilution that gave the same O.D. value as the mean of the control samples plus three standard deviations from the mean.

C) The mean GnRH-specific antibody titres are considerably lower in the group of mice passively immunised with RH. The error bars correspond to one standard error of the mean.
Figure 4.5
Testosterone levels in sera from BALB/c mice passively immunised with GnRH-specific rabbit antisera

A) Testosterone levels in the serum of each mouse (log scale). P1a-e were injected with R1 serum, P2a-e with RH and P3a-e with normal rabbit serum.

B) Mean testosterone levels within each group (linear scale). The error bars correspond to one unit of standard error. The differences between the passively immunised mice and the mice given normal rabbit serum are not significant (p>0.05 in both cases).
Testosterone measurements were done using a commercial RIA (DPC). Although there seemed to be a trend toward lower testosterone levels in the passively immunised mice than in controls the difference was not significant (p>0.05 for both P1 and P2 when compared to control group, P3). As seen before (figure 4.1) the testosterone levels varied widely between individual animals, with wide-ranging values within each group (figure 4.5).

The mice were sacrificed, the testes were collected, weighed and then flash-frozen in liquid nitrogen in a freezing medium. The testes of the passively immunised mice showed a significant reduction in weight (p=0.0002 for P1 and p=0.0066 for P2 when compared to P3, see figure 4.6). The frozen samples were sectioned in a cryostat and then fixed and stained with haemotoxylin and eosin stain. As can be seen in the pictures of the testis sections there are mature spermatocytes in the lumens of the seminiferous tubules (figure 4.7).
Figure 4.6
Testes weight of BALB/c mice passively immunised with GnRH-specific rabbit antibodies

The mice in both group P1 (passively immunised with R1) and P2 (passively immunised with RH) had significantly (p<0.05)* reduced testes weight compared to the control group P3. The error bars correspond to one unit of SE.
Figure 4.7
Section of a seminiferous tubule from a mouse passively immunised with anti-GnRH serum (mouse P1b)

The section was prepared by freezing the specimen in liquid nitrogen in a freezing medium and then sectioning in a cryostat (6 µm). The sections were then fixed, stained with haemotoxylin and eosin, making the cell nuclei prominent. Mature spermatozoa can be detected in the tubule lumen (indicated with arrows).
4.3 Discussion

Antibodies against reproductive hormones have the potential to neutralise the endogenous hormone and affect the reproductive status of an animal. Administration of GnRH-specific antisera can indicate the biological effect on the reproductive system, and on hormones under the control of GnRH, that might occur following a successful active immunisation against the GnRH peptide.

Previous experiments have shown that the immune response to different constructs of GnRH can vary greatly depending on which carrier molecule is used, what adjuvant and which species is immunised (see table 1.1). The immune responses of individual animals are often wide-ranging. Administration of pre-formed antibodies should show less variability between individuals but rather relate to the antibody levels and specificity of the antisera.

GnRH conjugated to the carrier molecules KLH and TT successfully induced an immune response against the GnRH peptide in rabbits. The GnRH-specific antibody titre was however higher in the rabbits immunised with GnRH-TT (R1 and R2) than the ones immunised with GnRH-KLH (R3 and R4). Rabbit serum specific for GnRH raised against a multivalent construct of GnRH with KLH as a carrier was given to us by Dr. R Meloen (RH). The GnRH-specific antibody titre in RH was found to be considerably lower in this specific assay system than the antibody titre in R1. The two antisera were used to immunise pre-pubertal male BALB/c mice.

The serum testosterone levels in unimmunised male BALB/c mice were measured at two-weekly intervals from the age of two weeks until the mice had reached 12 weeks of age, by which time they were all well past puberty. The level of testosterone seemed to peak at around 6-8 weeks of age, which coincided with the time the mice were due to reach puberty. The passive immunisations were therefore planned to start with 3-4 week old mice and carry on until the mice had passed puberty, around 9-10 weeks of age. The injections were done every week to compensate for the catabolism of the antibodies.
Raising Antibodies and Passive Immunisations

Rabbit GnRH-specific antibody titres in the sera of the passively immunised mice were determined by ELISA and the antibody titres in relation to the titres in mice immunised with non-specific rabbit serum. The mice in group P1 (passively immunised with R1 serum) had higher titres than the mice in group P2 (immunised with RH). This is most probably due to the lower titre of GnRH-specific antibody in RH than in R1, although different rates of catabolism of the antibodies could also influence the levels.

Passive immunisations with GnRH-specific antisera have been shown to reduce testosterone levels in sexually mature rats down to a third of the levels before immunisations (Hauger et al., 1977). The testosterone levels in the mouse sera in the present study were measured using a RIA and showed lower mean testosterone levels in the passively immunised groups than in the control group although the differences were not significant. The Pearson’s correlation coefficient for antibody levels compared to serum testosterone levels was found to be $r = -0.155$, which is not significant ($p>0.05$; the critical value of $r$ is ±0.666 at the 0.05% confidence level, n=9). The variance within each group is large so it is difficult to conclude on any direct effects of anti-GnRH antibodies on testosterone levels from these measurements.

Experiments using GnRH antagonists to inhibit the activity of GnRH in vivo have shown that there is significant species variability in the sensitivity to GnRH antagonists. Rats seem to be more sensitive to the antagonists than mice and rabbits for example, with serum testosterone and LH levels significantly reduced within 24 hours of an injection of GnRH-specific antisera (Limonta et al., 1985, Ultee-van Gessel et al., 1989, Sundaram et al., 1984). Suppression of meiosis of germ cells and reduction of testosterone and LH levels in male mice has been achieved by a GnRH-antagonist, but at high levels and constant administration over long periods of time (Szende et al., 1990). From this it could be concluded that mice are less responsive to GnRH inhibition or neutralisation, at least for the effect on serum testosterone and gonadotropin levels.

Although the effect of passive immunisation on testosterone level was not apparent, the effect on testis weight was. The testes of the immunised mice weighed significantly less than the testes of control animals ($p<0.05$ for both groups), in some cases around half the normal weight. In spite of this biological difference in the
immunised animals, spermatogenesis seemed to have developed normally. There were mature sperm in sections from all the immunised mice. In addition there was no sign of premature release of meiotic spermatocytes into the lumen of the seminiferous tubules (see section 5.2.4).

Hauger and colleagues found that immunoneutralisation of GnRH in male rats resulted in considerable reduction of LH and FSH values (below 10% of normal values) but the effect on testosterone was not as striking (Hauger et al., 1977). They proposed that the ether anaesthesia might have had an effect in raising the testosterone levels. When mice were decapitated without anaesthesia the testosterone levels were found to be similar to those found in other GnRH neutralisation experiments (Bercu et al., 1977). Stress factors such as anaesthesia and multiple immunisations can indeed influence the levels of testosterone and also the levels of antibodies produced in an active immunisation with an immunogen.

The reduction of weights of the mouse testes in the passively immunised mice is clear evidence of a biological effect of the GnRH antisera. Although testes weights and testosterone levels are usually equally affected by complete neutralisation of GnRH, testosterone levels are frequently variable, even in untreated animals and, at least in studies on piglets, the effect of GnRH neutralisation, by active immunisation against GnRH, seems to affect testosterone levels later than testes weights (Meloen et al., 1994). The fact the testosterone levels in the passively immunise mice remained unaffected and were highly variable and that spermatogenesis had not been disrupted indicated that GnRH had not been completely neutralised.

One of the reasons for the failure of complete neutralisation of endogenous GnRH could be that GnRH-specific antibody levels in the rabbit antisera were too low. R1 rabbit serum had a considerably higher GnRH-specific antibody titre (about 8 times higher) than RH and mice passively immunised with R1 did indeed show more reduction of testis weight than mice passively immunised with RH. Also antibody against any hormone can stabilise the hormone by reducing its degradation rate and therefore making it more efficient in activating its receptor. There is however no evidence for such an up-regulatory effect on GnRH by the anti-GnRH antisera in this case.
Chapter 5  Immunisations with Constructs of GnRH
5.1 Introduction

Immunological castration of male rodents is possible by immunisation with GnRH constructs. A successful GnRH-specific antibody response normally requires conjugation of GnRH to a carrier molecule, administration in strong adjuvants or multiple injections of the immunogens. The effects of GnRH immunisations are very varied between different experiments. In some sets of immunisations there is considerable atrophy of the testes and other reproductive organs and spermatogenesis is abolished, but these effects can differ drastically between individuals receiving the same treatment (e.g. Fraser et al., 1974, Morrison et al., 1987, Quesnell et al., 2000, Upadhyay et al., 1989).

To evaluate the effect of immunisations against GnRH on the reproductive system the state of spermatogenesis is most indicative of intact fertility. The morphology of the testes and production of androgens are also important factors affecting reproduction and signs of the state of fertility.

The testis is compartmentalised into four distinct parts. The vascular compartment contains the blood vessels of the testes, the interstitial compartment surrounds the seminiferous tubules and consists of Leydig cells, connective tissue and lymphatics. The basal compartment is the barrier region between the adluminal region and the interstitium. The adluminal and the basal regions are within the seminiferous tubules. The barrier comprising the ‘blood-testes barrier’ consists of multiple layers of gap and tight junctions encircling each Sertoli cell, linking them firmly together and preventing diffusion between interstitium and lumen.

Spermatogenesis is a complex and highly organised procedure. In order to understand the subtle morphological changes that might occur after androgen depletion following GnRH immunisation the process will be explained briefly in the next section.
5.1.1 Spermatogenesis

Germ cells do not divide until after puberty. The spermatogonia divide mitotically. They are divided into type A (A0, A1, A2, A3 and A4), intermediate and type B spermatogonia. Type A divide by mitosis and produce other spermatogonia and type B differentiate into primary spermatocytes. Spermatocytes divide by meiosis to form the haploid spermatids. The spermatids undergo spermiogenesis, i.e. the formation of a highly differentiated spermatozoon, with a tail and acrosome. They are then released into the seminiferous tubule lumen in a process called spermiation and are washed down the length of the tubules by the testicular fluid, produced by Sertoli cells. Spermatogenesis occurs in highly regulated species-specific cycles. Each spermatogonium produces offspring that are linked together by thin cytoplasmic bridges and progress through mitotic and meiotic divisions at the same and constant rate. Once a type A spermatogonium has started to divide and differentiate through the spermatogenic pathway another type A cell does not enter the pathway until after a certain constant, also species-specific, amount of time.

In a transverse section of a seminiferous tubule cells at 4-5 (depending on the species) different stages of the spermatogenic cycle can be observed. As the spermatogenic cycle and the development of a type A spermatogonium to fully mature spermatozoa are both constant in length, cells in successive cycles always develop in parallel. The cycles are divided into stages I-XIV, depending on where in the process of spermatogenesis the spermatogonia are in one cross-section of a tubule. The Sertoli cells are thought to organise the spermatogenic activity by mediating the effects of hormones and other factors on the spermatocytes. The Leydig cells produce steroids such as testosterone which enters the seminiferous tubules where it is bound by the Sertoli cells, thereby supporting spermatogenesis.

Testosterone has an effect on protein secretion of the tubules, especially at stages VI-VIII (McKinnell and Sharpe, 1992, Sharpe et al., 1992, Sharpe et al., 1993). There is also a stage dependent increase of lumen size at stages VII-VIII (Sharpe, 1989) which is likely to be a direct consequence of increased production of seminiferous fluid by the Sertoli cells. Testosterone withdrawal by either injection of ethane dimethane sulphonate (EDS), which destroys Leydig cells, or by hypophysectomy in adult rats
resulted in a reduction of lumen size at stage VII and a 50% reduction in production of seminiferous tubule fluid per testis (O'Leary et al., 1987, Ghosh et al., 1992). Interestingly the same is not true regarding immature rats. Seminiferous tubule fluid production only starts between days 15-20 after birth in the rat, the same time as the inter-Sertoli cell junctions start to develop, forming the blood-testis barrier (Jegou et al., 1982).

5.1.2 Relaxin-like factor (RLF) expression

The development of the interstitial compartment in the mouse testis is characterised by the formation of first a foetal Leydig cell population, producing large amounts of testosterone and then replacement postnatally by a population of differentiated, adult Leydig cells with LH receptors and active steroidogenesis, commencing at puberty (O'Shaughnessy et al., 1998). RLF, which is also known as the Leydig cell insulin-like (Ley-I-L) peptide, can be used as a marker of Leydig cell differentiation status. It is produced in a constitutive manner within both foetal and adult Leydig cells, but in the foetal cells its expression is independent of gonadotrophin stimulation (O'Shaughnessy et al., 1998). RLF is expressed in foetal Leydig cells and immediately post-natally in both the hypogonadal (hpg) and the wt mouse, whereas pre-pubertal Leydig cells exhibit only low levels of RLF expression in both types of mouse (Balvers et al., 1998). Testes RLF expression is upregulated during puberty in wt mice but not in hpg mice. By day 15 the Sertoli cell tight junction barrier is in place and RLF immunostaining is limited to the differentiated Leydig cells. By the end of puberty (around day 35 and onwards) the cell staining for RLF is more homogenous and strictly limited to the Leydig cells (Balvers et al., 1998).

Pusch and colleagues found that RLF gene expression is upregulated post-pubertally in the male mouse in response to raised levels of androgens. The lack of postmeiotic spermatocytes in the azoospermic mutant (w/w') mouse did not reduce the levels of RLF, while mutant Leydig cell lines, incapable of testosterone production, show reduced levels of RLF expression compared to primary Leydig cells (Pusch et al., 1996).
Immunisations with constructs of GnRH

The mouse RLF cDNA was cloned into a pMOS-Blue (Amersham Pharmacia Biotech) plasmid vector and sequenced (Pusch et al., 1996). The cDNA was given to us by Dr. Richard Ivell (IHF, University of Hamburg) and used to make a radiolabelled probe (a 283-bp restriction fragment) specific for the RLF sequence.

5.1.3 Multiple antigen peptide systems

In addition to the conjugates of GnRH and the carrier molecules TT and Hsp70 and the phage display of GnRH, a multiple antigen peptide (MAP) system was also used to immunise mice. GnRH can be made more immunogenic by conjugating multiple copies of it, for example using tandem linear repeats (Meloen et al., 1994). A MAP (Tam, 1988, Tam and Zavala, 1989) is based on an inert lysine core with radial branches onto which peptides are attached (see figure 5.1). This macromolecule usually has either four or eight peptides connected to the core and a unique threedimensional structure. It is potentially a much better immunogen than the peptide on its own and could eliminate the need for a large foreign carrier protein.

![Figure 5.1](image_url)

Figure 5.1
A multiple antigen peptide (MAP) system

A schematic representation of a tetravalent MAP with four copies of GnRH anchored to its amino terminals.

MAPs can contain both B and T cell epitope peptides to enhance the immune response against either peptide. Tam and Lu showed that by including residues 12-26 of the pre-S(2) region of the middle protein of the hepatitis B surface antigen along with a determinant of the S protein, induced an immune response against the otherwise poorly immunogenic S protein determinant and antibodies were made against both peptides that also recognised the whole S protein and the middle protein (Tam and Lu, 1989). In this way a MAP can be used to produce an epitope-specific vaccine against short
peptides corresponding to the epitopes as well as circumventing the need for large molecular weight carrier molecules.

Two different types of MAPs were synthesised incorporating the amino acid sequence for GnRH. One was an octavalent lysine core with eight copies of the GnRH peptide anchored to it (synthesised by Sigma). The other had a tetravalent core but incorporated a T helper cell epitope peptide in tandem with the GnRH peptide on its branches (synthesised by Dr. K.S. Iyer, Institute for Research in Reproduction, Mumbai, India). The amino acid sequence bound to a tetravalent lysine core was NHWSTGLAPGGGLSNIKGVIVHALNGV-MAP.

An irrelevant MAP including a random sequence was used as a control immunogen. The random peptide sequence bound to an octavalent lysine core was ILRFRYYL-MAP. The GnRH-MAP without the T helper epitope and the irrelevant MAP were injected in a mixture of PBS and a T helper epitope peptide (LSEIKGVIVHRLEGV), derived from the measles fusion protein (kindly given to us by Professor M. Steward), in Ribi (MPL+TDM) adjuvant. The GnRH-Th-MAP was injected in a mixture of PBS and Ribi.

Male mice were immunised with a number of GnRH constructs. GnRH was conjugated to TT and Hsp70, displayed on the surface of a bacteriophage, and as MAP constructs.
5.2 Results

5.2.1 Immunisations with different constructs of GnRH

Two sets of immunisation experiments were done using various different GnRH constructs. In the first set six groups of five male BALB/c mice were immunised. The initial injections were done on 3-4 week old immature mice and additional boosts were given at 2 and 4 weeks later. Each mouse was injected with a total volume of 200 µl of immunogen diluted in Ribi adjuvant i.p. The mice were sacrificed when they were 9-10 weeks of age, exsanguinated, weighed and relevant tissues harvested. In the second set five groups of five male BALB/c mice were immunised using the same schedule as above. The groups are listed in table 5.1 below. In each set one group of mice was injected with the adjuvant mixed with PBS as a negative control.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice</th>
<th>Immunised with</th>
<th>Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M1a-M1e</td>
<td>GnRH-MAP and Th</td>
<td>Ribi</td>
</tr>
<tr>
<td>2</td>
<td>M2a-M2e</td>
<td>Irrelevant MAP and Th</td>
<td>Ribi</td>
</tr>
<tr>
<td>3</td>
<td>M3a-M3e</td>
<td>GnRH-TT</td>
<td>Ribi</td>
</tr>
<tr>
<td>4</td>
<td>M4a-M4e</td>
<td>GnRH displayed on phage</td>
<td>Ribi</td>
</tr>
<tr>
<td>5</td>
<td>M5a-M5e</td>
<td>wt phage</td>
<td>Ribi</td>
</tr>
<tr>
<td>6</td>
<td>M6a-M6e</td>
<td>PBS (control)</td>
<td>Ribi</td>
</tr>
<tr>
<td>7</td>
<td>M7a-M7e</td>
<td>GnRH-Hsp70</td>
<td>ICF</td>
</tr>
<tr>
<td>8</td>
<td>M8a-M8e</td>
<td>GnRH-Hsp70</td>
<td>Ribi</td>
</tr>
<tr>
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<td>M9a-M9e</td>
<td>GnRH-MAP-Th</td>
<td>Ribi</td>
</tr>
<tr>
<td>10</td>
<td>M10a-M10e</td>
<td>GnRH-MAP and Th</td>
<td>Ribi</td>
</tr>
<tr>
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<td>M11a-M11e</td>
<td>GnRH-MAP and Th</td>
<td>Ribi</td>
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<tr>
<td>12</td>
<td>M12a-M12e</td>
<td>PBS (control)</td>
<td>Ribi</td>
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Table 5.1
Groups of mice immunised with various different GnRH constructs and the adjuvants used
(ICF = Incomplete Freund’s adjuvant)

Ribi adjuvant was used in all the immunisations above except for one group immunised with GnRH-Hsp70 mixed in ICF. Ribi was chosen as it is an oil-in-water emulsion containing MPL and TDM, powerful immunostimulants that cause little or no detectable toxic or allergic reactions (e.g. Johnson et al., 1987, Ulrich and Myers, 1995). ICF is completely devoid of active adjuvanticity, but acts as a depot of immunogen as a water-in-oil (paraffin oil and mannide monooleate) emulsion. ICF
was used to compare the carrier efficacy and adjuvanticity of Hsp70 with or without added adjuvant.

5.2.2 Antibody titres

The GnRH-specific antibody endpoint titres in the mouse sera were determined by ELISAs. Each group was compared to the mean of the relevant control group. The endpoint titre was defined as the serum dilution which gave an absorbance signal equal to the mean plus three standard deviations from the mean of the control group (mice immunised with PBS).

Four groups were immunised with MAPs. Groups 1 and 11 were immunised with GnRH-MAP along with a T helper (Th) cell epitope. Group 10 was immunised with a GnRH-MAP with the T helper cell epitope covalently linked to the MAP and in tandem with GnRH. Group 2 was immunised with an irrelevant MAP, bearing the same core lysine group but not the GnRH peptide. The results of the ELISAs done on sera from mice in groups 1, 2, 10 and 11 are shown in figures 5.2-4. The endpoint titre for each animal in the three groups is summarised in figure 5.5. The three GnRH-MAP immunised groups did not differ significantly (p>0.05) in their antibody titres, although the individual mice with the highest values were those immunised with the tandem construct of GnRH-MAP-Th.
Figure 5.2
Antibody titration curves of mice immunised with GnRH-MAP along with a measles-derived Th peptide (group 1)

A) GnRH-specific antibody titration curves. ELISA plates were coated with GnRH, the mouse serum allowed to bind to the peptide and then detected by mouse Ig-specific sera conjugated to alkaline phosphatase.

B) MAP-specific antibody titration curves. ELISA plates were coated with an irrelevant MAP to detect antibodies against the lysine core structure of the MAP.
Immunisations with constructs of GnRH

A) Mice in group 10 were immunised with GnRH-MAP covalently linked to a measles-derived T helper cell epitope peptide. GnRH-specific antibodies in the mouse sera were detected by alkaline phosphatase-conjugated anti-mouse antibody on a GnRH coated ELISA plate.

B) Mice in group 11 were immunised with GnRH-MAP along with a measles-derived Th peptide. GnRH-specific antibodies were detected on a GnRH coated ELISA plate as in A.
GnRH-specific antibodies in mice immunised with an irrelevant MAP construct (group 2)

Figure 5.4
GnRH-specific antibody titration curves of mice immunised with an irrelevant MAP along with a measles-derived Th peptide (group 2)

The GnRH-specific antibodies in the mouse sera were measured on GnRH coated ELISA plates. None of the mice showed GnRH-specific antibodies.
Immunisations with constructs of GnRH

**Figure 5.5**
The endpoint antibody titres (log scale) of mice immunised with the various MAPs

A) The endpoint titre of GnRH-specific antibodies in each animal.
B) The mean endpoint titre for each group. The error bars indicate 1 standard error from the mean.
Endpoint antibody titres were similarly measured for mice immunised with GnRH-TT (figure 5.6), GnRH displayed on the surface of bacteriophages (figure 5.7) and GnRH-Hsp70 (figure 5.8). Four out of five mice immunised with GnRH conjugated to TT carrier showed detectable levels of GnRH-specific antibodies (figure 5.6 A) but all of them showed high TT-specific antibody levels (figure 5.6 B). All of the mice immunised with GnRH expressed on the surface of a filamentous bacteriophage showed detectable GnRH-specific antibody levels (figure 5.7 A) and none of those injected with wt bacteriophage had GnRH-specific antibodies in their sera (figure 5.7 B). All the animals immunised with GnRH-Hsp70 (groups 7 and 8) showed an anti-GnRH antibody response regardless whether the immunogen was administered in Ribi adjuvant or in ICF. The mice in these groups also showed the highest mean antibody levels of all the groups immunised with the different GnRH constructs. The endpoint antibody titres are summarised in figure 5.9.

Hsp70-specific antibody titres were measured in the GnRH-Hsp70 immunised groups (groups 7 and 8). The results are shown in figure 5.10. Antibody levels in sera from mice immunised with hCG conjugated to Hsp70, administered in Ribi (group 9) were also plotted on the graph as a comparison. None of the mice immunised with GnRH conjugated to Hsp70 (either in ICF or Ribi) showed any Hsp70-specific antibodies. Mice immunised with the hCG-Hsp70 construct on the other hand showed high levels of Hsp70-specific antibodies (figure 5.10).
Figure 5.6
Antibody titration curves in GnRH-TT immunised mice (group 3)

A) GnRH-specific antibody levels were measured on GnRH coated ELISA plates. 
B) TT-specific antibody levels were measured on TT coated ELISA plates.
Figure 5.7
GnRH-specific antibody titration curves in mice immunised with GnRH displayed on the surface of bacteriophage (A) and with wt bacteriophage (B).

The antibodies were measured on GnRH coated ELISA plates.
Immunisations with constructs of GnRH

**A**

GnRH-specific antibodies in mice immunised with GnRH-Hsp70 in ICF (group 7)

![Graph A](image)

**B**

GnRH-specific antibodies in mice immunised with GnRH-Hsp70 in Ribi (group 8)

![Graph B](image)

Figure 5.8
GnRH specific antibody titration curves in mice immunised with GnRH conjugated to Hsp70 in either ICF (A, M7a-M7e) or Ribi adjuvant (B, M8a-M8e)
Figure 5.9
Summary of GnRH-specific antibody endpoint titres

A) GnRH-specific antibody titres in individual animals immunised with GnRH constructs.

B) Mean GnRH-specific antibody titres in each group. The error bars indicate one standard error from the mean.
Figure 5.10
Mean Hsp70-specific antibody titration curves in mice immunised with Hsp70 conjugates

Mean antibody titration curves for mice immunised with GnRH-Hsp70 in ICF (group 7), GnRH-Hsp70 in Ribi (group 8), hCG-Hsp70 in Ribi (group 9) and controls (group 12) were plotted against serum dilutions. None of the mice immunised with the GnRH-Hsp70 constructs show any Hsp70-specific antibodies. The hCG-Hsp70 immunised mice show high Hsp70-specific antibody titres.
5.2.3 Testosterone levels

Serum testosterone levels were measured by RIA. The results for each set of experiments are shown in figures 5.11 and 5.12. The testosterone levels showed great variation within each group, especially in the two groups immunised with GnRH-Hsp70 (groups 7 and 8). Compared to the control groups immunised with PBS (groups 6 and 12) the only group that showed a significant reduction in testosterone levels (p = 0.0071) was the one immunised with GnRH-MAP in Ribi adjuvant (group 1).

To check whether the variability of testosterone levels was due to variability in the antibody response, the correlation coefficient for testosterone levels to GnRH-specific antibody levels was calculated. The GnRH-antibody levels were calculated as the O.D. of a 1:100 dilution of serum on an ELISA multiplied by the O.D. for the controls at the same dilution. The Pearson's correlation coefficient was found to be $r = -0.361$, which is statistically significant (p<0.05; the critical value of r is ±0.294 at the 0.05% confidence level, n=45). The testosterone levels were plotted as a function of the antibody levels (figure 5.13).
Figure 5.11
Testosterone levels (1) in serum of immunised mice (log scale)

A) Testosterone levels in the serum of individual animals.
B) Mean testosterone levels in each group. The error bar corresponds to one standard error of the mean. Levels are significantly lower in group 1 (MAP-GnRH) than in group 2 (irrelevant MAP), p=0.0127 but the difference between groups 1 and 6 (control group) is not significant (p>0.05).
Figure 5.12
Testosterone levels (2) in serum of immunised mice (log scale)

A) Testosterone levels in the serum of individual animals.
B) Mean testosterone levels in each group. The error bars correspond to one standard error of the mean.
Correlation between testosterone levels and GnRH-specific antibody levels (O.D. at a 1:100 dilution, multiplied by O.D. of controls at the same dilution) in immunised mice

Testosterone levels (logarithmic scale) are plotted as a function of GnRH-specific endpoint antibody titres (logarithmic scale). Groups are as before (see table 5.1). The Pearson’s correlation coefficient for antibody and testosterone levels in immunised mice is $r = -0.3614$, $p<0.05$ ($n=45$), which implies that there is significant correlation between the two factors.
5.2.4 Effects on the testes

The mice were dissected and their testes removed, freed of fat and weighed. One testis was flash frozen for RNA extraction and analysis of differential gene expression. The other was fixed and embedded in paraffin to use for histological analysis. In addition the urogenital complex, which includes auxiliary reproductive organs, such as the prostate and the seminiferous vesicles, was examined and graded.

The GnRH immunised animals did not differ in testes weight from the control groups (groups 6 and 12, immunised with PBS); two mice in group 7 (immunised with GnRH-Hsp70 in ICF) even seemed to have heavier testes on average than the control groups (figure 5.14) although the difference was not significant.

The auxiliary reproductive organs were severely atrophied in some of the immunised mice. The urogenital complex comprises the seminiferous vesicles that are highly dependent on local testosterone levels, the various parts of the prostate gland (anterior, dorsal and ventral prostates) along with the urethra and bladder. The complex is very small and covered in fat tissue. Weighing it was found to be an inaccurate way of evaluating its degeneration. It was therefore decided to grade the urogenital complex of each individual in the second set of immunisation experiments corresponding to its involution. Atrophied urogenital complexes were given a grade of 1 and fully mature complexes a grade of 3. Intermediates were given a grade of 2. The difference in size and appearance between atrophied and normal complexes are shown in figure 5.15 and the grades given to individual mice are shown in figure 5.16. Groups 8 and 10 (mice immunised with GnRH-Hsp70 in Ribi and GnRH-MAP-Th respectively) showed significant atrophy compared to control animals.
Immunisations with constructs of GnRH

Figure 5.14
Testes' weights (mg)

A) Weights of testes of individual mice
B) Mean weights of testes in groups of immunised and control mice. There is no detectable reduction in weight of testes in immunised mice. The error bars correspond to one standard error from the mean.
Immunisations with constructs of GnRH

The dorsal and ventral views of the urogenital complex from a fully mature male mouse

Figure 5.15
The urogenital complex in non-immunised and immunised mice

A) An enlarged dorsal view of the urogenital complex from a fully mature male mouse.
B) A ventral view of the complex.
C) An atrophied urogenital complex from a mouse immunised with GnRH-Hsp70 (M8a). Note the atrophied seminiferous vesicles (SV).
D) The difference in size of an atrophied urogenital complex compared to an unaffected complex (dissected from a mouse from the control group (M6b)). The most striking difference in size is in the SV. The atrophied urogenital complexes were given a grade of 1 and the fully developed a grade of 3, intermediates were given a grade of 2.

AP = Anterior Prostate, BI = Bladder, CG = Coagulation Gland, DP = Dorsal Prostate, Ur = Urethra, VP = Ventral Prostate.
Figure 5.16
The degree of atrophy in the urogenital complexes of the immunised animals

Each mouse was graded according to the status of its urogenital complex. An atrophied complex received a grade of 1 or 2 dependent on the degree of atrophy (see figure 5.13 C). A fully developed (normal) complex received a grade of 3. Any intermediates were given grade 2. Groups 8 and 10 of immunised mice both show significant (p =0.0040, 0.0081) atrophy compared to the control group (group 12).
Histological analysis was also done on the testis samples. The testes were fixed, sectioned and stained with haemotoxylin and eosin. Fully matured spermatocytes were detected in the seminiferous tubules in all the samples (see figure 5.17). There were however some morphological differences between the immunised and the control groups. These differences were evaluated by fitting a 10 by 10 graticule in one of the eyepieces of a Zeiss binocular microscope. The volume densities of the interstitial tissue and seminiferous tubules in the testes were found by counting the number of points over the respective structure (interstitium, cellular component and lumen) and dividing by the total points over the testicular tissue. The counts were done on random sections of testis, in triplicate for each animal (see figure 5.18). The results are summarised in figure 5.18 C.

There was a significant reduction of interstitium in the sections of testes from mice immunised with GnRH-Hsp70 in RIBI, GnRH-MAP and GnRH-Th-MAP (p=0.0009, 0.0319 and 0.0015, respectively).

Another morphological difference between immunised and control animals was the increased appearance of meiotic spermatocytes (i.e. not fully formed spermatozoa) released into the lumens of the tubules. The meiotic spermatocytes are round and have darkly staining nuclei (figure 5.19). The ratio of tubules that had immature spermatocytes released into their lumens to tubules with only fully mature spermatocytes released was assessed for each animal. Groups 7 and 8 (immunised with GnRH-Hsp70 in ICF and Ribi, respectively) showed a significant increase (p = 0.006 and p = 0.049) in meiotic cells released into the lumens of the seminiferous tubules.
Figure 5.17
A section through a seminiferous tubule (100 x magnification)

There are clearly mature spermatozoa being released into the lumen of the tubule. The Sertoli cells surround the spermatogonia in the cellular component of the tubule. Surrounding the tubule is the basal membrane and in between tubules, in the interstitium, there are blood vessels and Leydig cells.
Immunisations with constructs of GnRH

Figure 5.18
Morphology of the testes

Point counts to evaluate the ratio of lumen to interstitium to cellular component of the seminiferous tubules were done on each mouse sample in triplicate. The counts were done by placing a grid over the magnified section (B). The results for the GnRH immunised mice are shown in (C). There is a significant reduction* of interstitium in the immunised groups compared to the control group (p<0.05, see text).
Immunisations with constructs of GnRH

The dark arrows point out meiotic spermatids that have been prematurely released into the lumen of the tubule.

The ratio of tubules that had meiotic cells released into their lumens to tubules with only mature spermatocytes was evaluated for immunised and control mice. The mice in groups 7 and 8 (immunised with GnRH-Hsp70 in ICF and Ribi, respectively) had significantly higher numbers (p<0.05 in both cases) of these cells released than controls.

Figure 5.19
Meiotic cells released prematurely
5.2.5 RNA analysis of testosterone dependent gene expression

RLF mRNA 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 neutralising effects of the GnRH immunisations and the effects of altered testosterone levels in the testes. RNA slot blots were done using testes RNA and RNA from the seminiferous vesicles, heart muscle and liver as negative controls, as RLF expression has been detected exclusively in the testis and, at much lower levels, in the ovary in adult mice (Zimmermann et al., 1997). The RNA was blotted onto Hybond N nitrocellulose membranes and probed with an RLF-specific radiolabelled oligonucleotide probe. RNA loading was corrected for by subsequent detection of β-actin mRNA. The RNA blots were quantified by phosphorimager and the output for RLF divided by the output for actin for each sample. The procedure is schematised in figure 5.20.

RLF expression was significantly lower in groups 7, 8 and 10, i.e. mice immunised with GnRH-Hsp70 in ICF and in Ribi and GnRH-MAP-Th, with p=0.0004, p=0.0005 and p=0.002, respectively, than in the control group (see figure 5.21).
Extracted RNA was blotted onto a nylon membrane (Hybond N) using a commercial slot-blower (Biorad). Each RNA sample was blotted in duplicate.

The membranes were probed with a \(^{32}\text{P}\)-labelled RLF-specific probe and subsequently with a \(^{32}\text{P}\)-labelled actin-specific probe and quantified in a phosphorimager.

**Figure 5.20**

RLF expression

Total RNA was extracted from one testis and, as controls, from liver, heart and seminiferous vesicles from each animal and pooled for each group to evaluate the testosterone dependent expression of RLF.

The RNA (20 \(\mu\)g in duplicate) was blotted onto a nylon membrane (Hybond N) using a slot-blotter. The membrane was hybridised with a \(^{32}\text{P}\)-labelled RLF-specific probe and the signal quantified in a phosphorimager. The membrane was stripped and re-probed with a \(^{32}\text{P}\)-labelled \(\beta\)-actin-specific probe. The RLF expression was evaluated as RLF output divided by the actin output (after subtraction of the background output for either case).
Immunisations with constructs of GnRH

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<td>Negative controls</td>
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Figure 5.21
The relative levels of RLF in GnRH immunised mice

The RNA blots were quantified by phosphorimager. Mice immunised with GnRH-Hsp70 in ICF (group 7) and in Ribi (group 8) and GnRH-Th-MAP (group 10) show significant reductions of RLF levels compared to the control group (Group 12, p=0.0003, 0.0005 and 0.0020 respectively). The ‘negative controls’ are RNA extracted from seminiferous vesicles, heart and liver.
5.3 Discussion

Male mice were immunised with a number of different GnRH constructs. All the immunised groups responded with measurable GnRH-specific antibody titres, although there was variation within some of the groups. The groups with the most consistent and highest titres were those immunised with GnRH conjugated to Hsp70 in both ICF and RIBI. Hsp70 is a powerful immunostimulant that has its own adjuvanticity (e.g. Lehner et al., 2000, Lussow et al., 1990), as is noticeable by the high titres of GnRH-specific antibody responses in mice immunised with GnRH-Hsp70 without any active adjuvant, i.e. in ICF.

Testes' weights and testosterone levels were not significantly different in immunised mice compared to control mice, except in group 1, immunised with GnRH-MAP, where testosterone levels were reduced. Testosterone levels are often variable between individual animals receiving the same treatment (e.g. Schanbacher et al., 1983, Ladd et al., 1994, Meloen et al., 1994). There was however a significant correlation between antibody levels and serum testosterone, with lower testosterone in animals with higher antibody levels. The testes' weights did not show any such correlation. Carelli and colleagues unexpectedly found that there was an increase in testes' weights in mice following immunisations against GnRH (Carelli et al., 1982).

The biological effects of GnRH immunisations were evident in the changes in testes' morphology and in the atrophy of secondary reproductive organs. Immunisations using GnRH-Hsp70 and both GnRH-MAP constructs resulted in gross atrophy of the urogenital complex of some of the immunised mice and subtle differences in the histology of the testes. The compartmentalisation of the testis is highly regulated and in some of the immunised individuals there was significantly less interstitium than in the controls. The interstitial compartment is mainly Leydig cells and blood vessels and the Leydig cells are sensitive to changes in gonadotrophin levels as well as androgen levels.

The biological effects of all the immunisations are summarised in table 5.2.
### Table 5.2
A summary of the results of GnRH immunisations of mice

Antibody endpoint titres were calculated as described in section 5.2.2. Significant differences in testosterone levels, testis weights, volume density of interstitium, meiotic cells or RLF expression in immunised mice compared to control mice are coloured yellow.

NA = Non-applicable
The fact that serum testosterone levels seemed unaffected in all but one of the groups, even higher in some of the immunised mice than in controls, and that there was a negative correlation between antibody and testosterone levels, indicates that the neutralisation of GnRH by GnRH-specific antibodies was not complete. The reason for incomplete neutralisation of the peptide could be that GnRH-specific antibody levels were not high enough, or that the antibodies prolonged the life of the peptide and therefore made it more efficient at activating its receptor. Up-regulation of GnRH was not significant for any of the immunised groups, largely due to the great variation between individuals of the same group. GnRH neutralisation affects testosterone levels by reducing the levels of LH in serum. If GnRH neutralisation has not been complete the effect on LH might not be strong enough to affect Leydig cell production and secretion of testosterone. However the biological effects seen on the testis compartmentalisation and on the gross morphology of the secondary reproductive organs does point to a disruption of normal reproductive control by GnRH.

RLF expression is strongly dependent on testosterone levels. RLF expression was significantly down-regulated in immunised animals which would strongly indicate lowered testosterone levels although they were not low enough to disrupt spermatogenesis and affect fertility. The RLF expression could also be lower because of a reduction of volume density of Leydig cells in the testis. RLF expression is confined to the Leydig cells and they are in turn highly dependent on LH levels within the testis.
Chapter 6  Immunisations with constructs of hCG and oLH and cascade inhibition of the reproductive pathway
Immunisations with constructs of hCG and oLH and cascade inhibition

6.1 Introduction

HCG has been used in the past in immunisation experiments mainly on females as a possible immunocontraceptive vaccine with good results (reviewed in e.g. Talwar, 1997a). LH from various different species has been used in immunisations, although mainly on male animals. The interspecies variability of LH has been enough to produce high antibody titres and affect androgen production, spermatogenesis and morphology of the reproductive organs. Ovine LH emulsified in FCA has been used to immunise male rats, resulting in oLH-specific antibody responses that cross-react with rat LH and neutralise the endogenous LH of the animal (Awoniyi et al., 1989, Wakabayashi and Tamaoki, 1966).

GnRH and LH are two consecutive steps in the reproductive hormone pathway (see figure 1.5). GnRH immunisations were apparently unable to neutralise the production of LH completely, as judged by the testosterone levels in serum (Chapter 5). Neutralisation of both GnRH and LH at once might potentially have a greater effect on the reproductive system than neutralisation of either step on its own. Experiments with other vaccine systems have shown that immunological protection against a pathogen (for example the Malaria parasite) can be greatly enhanced if the vaccine is aimed at multiple stages of the pathogens lifecycle (Shi et al., 1999). In a similar manner the co-immunisation of animals against GnRH and LH could enhance the effect on the reproductive system and reduce the individual variation in the response seen in previous immunisation experiments.

Neutralisation of murine LH was attempted using either hCG conjugated to Hsp70, to produce a hCG-specific antibody response that could potentially cross-react with the endogenous LH and neutralise it, or oLH. There is ample evidence to show that oLH is highly immunogenic in rodents and oLH-specific antibodies have been shown to neutralise the endogenous hormone in vivo in rats (Wakabayashi and Tamaoki, 1966, Quadri et al., 1966, Hayashida, 1963, Awoniyi et al., 1989).

In the previous immunisations with different GnRH constructs (chapter 5) the highest GnRH-specific antibody titres were generated after immunisation with GnRH-Hsp70
Immunisations with constructs of hCG and oLH and cascade inhibition and GnRH-Th-MAP. These constructs were therefore chosen for a co-immunisation along with oLH to test the hypothesis that cascade inhibition is more efficient at affecting fertility of the mice than immunisation with either immunogen on its own.
6.2 hCG-Hsp70 conjugate

Five pre-pubertal male BALB/c mice were immunised with hCG conjugated to Hsp70 in Ribi adjuvant following the same immunisation protocol as for the GnRH immunised mice. The mice all responded with hCG-specific antibody titres that cross-reacted strongly with rat LH (figure 6.1). Testosterone levels were measured as before and are plotted in figure 6.2. No significant difference in testosterone levels was found between the immunised and the control mice. On dissection the testes were weighed (figure 6.3) and kept for either histological analysis or gene expression analysis. The testes of the hCG immunised mice were significantly lighter than the control group’s (p=0.0369) and the urogenital complex, including the seminiferous vesicles and the prostate were atrophied in four out of the five immunised mice (figure 6.4 A). The atrophy was however not the extent of atrophy as in some of the GnRH immunised mice.

Spermatogenesis did not seem to be affected by the hCG-Hsp70 immunisations. There were mature spermatozoa in the testes of all the immunised mice, but there were differences in the compartmentalisation of the testis. There was a significant reduction in the interstitial compartment of the immunised mice (p=0.0316) compared to the control group (figure 6.4 B). There was however not found to be a higher incidence of meiotic spermatocytes being released into the tubule lumens as had been found previously in mice immunised with the various GnRH constructs (figure 6.4 C).

Testosterone dependent RLF expression in the Leydig cells was evaluated by Northern hybridisation as before. There were no significant changes to the gene expression in the group of hCG-Hsp70 immunised animals (see figure 6.5).
Immunisations with constructs of hCG and oLH and cascade inhibition

Figure 6.1
Mice immunised with hCG-Hsp70

A) Rat LH-specific antibodies in serum from mice immunised with hCG-Hsp70 was measured by ELISA. The ELISA plate was coated with rat LH, probed with serum and then with mouse IgG-specific polyclonal antibody conjugated to alkaline phosphatase.

B) hCG specific antibodies in the same serum samples were measured by ELISA using the same method as in A) but with a hCG coated plate.

C) Endpoint antibody titres, both hCG and rat LH-specific, for the same group of mice. The endpoint titres were determined as the serum dilution with an O.D. value equal to the mean of the control samples (n=5) plus three standard deviations from the mean. The rat LH-specific antibody titres are higher than the hCG-specific when compared to the control group.
Figure 6.2
Testosterone levels (log scale)

A) Testosterone levels in individual animals.
B) Mean testosterone levels in both the hCG-Hsp70 immunised group and the control group (immunised with PBS). The difference between the two groups is not significant (p>0.05). The error bars are equivalent to one standard error.
Immunisations with constructs of hCG and oLH and cascade inhibition

Figure 6.3
Weights of testes

A) Weights of testes of individual mice immunised with hCG-Hsp70 and control mice (immunised with PBS).
B) Mean weights of testes in either group. The error bars indicate one standard error from the mean. The immunised mice have significantly lighter testes than the control mice (p=0.0369).
Immunisations with constructs of hCG and oLH and cascade inhibition

**Figure 6.4**

Morphology of the testes and the secondary reproductive organs

A) The state of atrophy of the urogenital complexes of immunised and control mice. All but one of the immunised mice show incomplete atrophy of the structure.

B) Point counts. The compartmentalisation of testis was evaluated. The interstitium in the immunised mice was significantly reduced compared to the control mice (p=0.0319).

C) Meiotic cells released prematurely into the seminiferous tubule lumens. The difference between the immunised and the control mice is not significant (p>0.05).
Figure 6.5
RLF expression

The testosterone dependent RLF expression in hCG-Hsp70 immunised mice and in control mice. There was not a significant difference between the two groups.
6.3 oLH

Five male BALB/c mice were immunised with oLH in Ribi adjuvant. The mice all responded with oLH-specific antibodies and three mice sera out of five cross-reacted with rat LH the other two showed marginal levels of cross-reactivity when tested on rat LH coated ELISA plates (figure 6.6). Serum testosterone levels were not found to be different in immunised animals compared to controls (figure 6.7).

The testes were weighed and dissected but there were no detectable differences in the testis weight or morphology from the control animals (see figures 6.8 and 6.9 A). The urogenital complex that had in other immunised groups been severely atrophied was unchanged in the oLH immunised group (see section 6.4.2). Spermatogenesis was also unaffected, mature spermatozoa were seen in all the testis sections looked at from the oLH immunised mice, as was RLF expression in the Leydig cells (see figure 6.9 B).
Immunisations with constructs of hCG and oLH and cascade inhibition

Figure 6.6
Mice immunised with oLH

A) Rat LH-specific antibodies in the sera of mice immunised with oLH were measured on ELISA using rat LH coated ELISA plates and probing first with mouse sera and then with mouse IgG specific rabbit polyclonal antibody, conjugated to alkaline phosphatase.

B) Ovine LH-specific antibodies were measured in the same manner using oLH coated ELISA plates.

C) Endpoint antibody titres, rat LH and oLH-specific, for the same group of mice. The oLH antibody titres are higher than the rat LH-specific titres.
Immunisations with constructs of hCG and oLH and cascade inhibition

Figure 6.7
Testosterone levels

A) Testosterone levels in individual mice immunised with oLH in Ribi and controls (immunised with PBS in Ribi)
B) Mean testosterone levels in oLH immunised mice and controls. The error bars are equal to one standard error from the mean.
Immunisations with constructs of hCG and oLH and cascade inhibition

Figure 6.8
Weights of testes

A) Weights of testes of individual mice immunised with oLH and control mice (immunised with PBS).

B) Mean weights of testes in both groups. The error bars indicate one standard error from the mean. There is no significant difference in testes weights between the two groups.
A) The compartmentalisation of the testis was evaluated by performing point counts over sections of testis. The volume density of the interstitium is unaffected by the oLH immunisations.

B) RLF expression in oLH immunised mice did not show significant differences from the control group.
6.4 Co-immunisation with GnRH constructs and oLH

The theory that cascade inhibition of the reproductive pathway, i.e. neutralisation of GnRH and LH, two consecutive steps in the hormonal cascade, is more efficient at blocking spermatogenesis and affecting fertility than the neutralisation of either factor in its own was tested by co-immunising mice with GnRH-Hsp70 and oLH, or with GnRH-Th-MAP and oLH. Additionally one group was immunised with oLH and passively immunised with GnRH-specific rabbit antiserum, as passive immunisations had previously resulted in reduced testis weights. Relevant control groups were included, with each immunogen on its own, one without immunogen (mice immunised with PBS) and one passively immunised with non-specific rabbit serum.

6.4.1 Antibody titres

Five male BALB/c mice were immunised in each group. The groups are listed in table 6.1 below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice</th>
<th>Immunised with</th>
<th>Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>M13a-M13e</td>
<td>oLH</td>
<td>Ribi</td>
</tr>
<tr>
<td>14</td>
<td>M14a-M14e</td>
<td>GnRH-Hsp70</td>
<td>Ribi</td>
</tr>
<tr>
<td>15</td>
<td>M15a-M15e</td>
<td>GnRH-Hsp70 and oLH</td>
<td>Ribi</td>
</tr>
<tr>
<td>16</td>
<td>M16a-M16e</td>
<td>GnRH-Th-MAP</td>
<td>Ribi</td>
</tr>
<tr>
<td>17</td>
<td>M17a-M17e</td>
<td>GnRH-Th-MAP and oLH</td>
<td>Ribi</td>
</tr>
<tr>
<td>18</td>
<td>M18a-M18e</td>
<td>Passively immunised with GnRH antiserum</td>
<td>None</td>
</tr>
<tr>
<td>19</td>
<td>M19a-M19e</td>
<td>Passively immunised with GnRH antiserum and oLH</td>
<td>None, oLH in Ribi</td>
</tr>
<tr>
<td>20</td>
<td>M20a-M20e</td>
<td>Non-specific rabbit serum</td>
<td>None</td>
</tr>
<tr>
<td>21</td>
<td>M21a-M21e</td>
<td>PBS</td>
<td>Ribi</td>
</tr>
</tbody>
</table>

Table 6.1
Mice immunised with different constructs of GnRH, GnRH and oLH together and oLH on its own

The GnRH-specific antibody titres were found to be high in all the actively immunised groups (groups 14-17), higher than in the passively immunised groups, receiving injections of GnRH-specific rabbit antiserum (R9) every week. The GnRH-specific antibody levels for a serum dilution of 1:100 are shown in figure 6.10.
Immunisations with constructs of hCG and oLH and cascade inhibition

Figure 6.10
GnRH specific antibody levels in 1:100 dilutions of sera

A) GnRH specific antibody levels in individual animals (measured as absorbance at 405 nm)
B) Mean GnRH specific antibody levels in the groups immunised with GnRH constructs and with GnRH constructs and oLH.
The highest GnRH-specific antibody levels were in the groups immunised with GnRH-Hsp70, GnRH-Hsp70 + oLH, GnRH-Th-MAP and GnRH-Th-MAP + oLH.

LH-specific antibody titres on the other hand were high in the group immunised only with oLH (group 13), but were weakly or not at all detectable in mice immunised with a mixture of GnRH construct or GnRH-specific antiserum and oLH (see figure 6.11). The antibodies in the sera of mice from group 13 cross-reacted with rat LH as well as oLH (see figure 6.6).

**6.4.2 Testosterone levels, testes morphology and atrophy of the urogenital complex**

Testosterone levels were reduced in five out of the seven immunised groups (see figure 6.12). The oLH immunised mice did not show a significant difference in testosterone levels compared to the PBS immunised control group. Neither did the GnRH-Hsp70 and oLH immunised group. The GnRH-Hsp70 immunised mice (group 14) and the GnRH-Th-MAP and GnRH-Th-MAP + oLH immunised mice (groups 16 and 17) did however have significantly lower levels of testosterone, p=0.0055, 0.0066 and 0.0055 respectively. The passively immunised mice, both with and without active immunisation with oLH had lower levels of testosterone, p=0.0141 and p=0.0094 for either group.

Dissection of the mice revealed severe atrophy of the urogenital complex in some of the immunised groups (see figure 6.13 A). The groups most affected were groups 14, 16 and 17, i.e. those immunised with GnRH-Hsp70, GnRH-Th-MAP and GnRH-Th-MAP along with oLH. All the animals in these groups showed complete atrophy of the complex. Interestingly the GnRH-Hsp70 and oLH immunised mice showed only incomplete atrophy along with the passively immunised mice. The mice immunised with oLH did not show any difference as mentioned in section 6.3.

The weights of the testes were significantly reduced in the mice of the same groups that had completely atrophied urogenital complexes (p=0.0158, 0.0007 and 0.0066 for groups 14, 16 and 17 respectively), see figure 6.13 B. In addition testes weights of the passively immunised mice (group 18) were also significantly lighter (p=0.0007).
Immunisations with constructs of hCG and oLH and cascade inhibition

Figure 6.11
Ovine LH-specific antibodies

A) Ovine LH-specific antibody titration curves. The antibody titres were measured on ELISA plates coated with oLH and detected by mouse IgG-specific polyclonal rabbit antibody, conjugated to alkaline phosphatase (except for the passively immunised mice were rabbit IgG-specific polyclonal goat antibody, conjugated to alkaline phosphatase, was used as secondary antibody).

B) Ovine LH-specific endpoint antibody titres. The highest titres were in the oLH immunised mice. The mice immunised with a mixture of GnRH construct and oLH (groups 15 and 17) did not show high antibody titres, neither did the group passively immunised with GnRH specific serum and actively immunised with oLH.
A) Testosterone levels in individual animals (log scale).

B) Mean testosterone levels in immunised groups. The error bars correspond to one standard error from the mean. Five out of the seven treated groups showed significantly lower testosterone levels compared to the control group (group 21).
Immunisations with constructs of hCG and oLH and cascade inhibition

A  
**Urogenital complex**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice Immunised with</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>oLH</td>
</tr>
<tr>
<td>14</td>
<td>GnRH-Hsp70</td>
</tr>
<tr>
<td>15</td>
<td>GnRH-Hsp70 and oLH</td>
</tr>
<tr>
<td>16</td>
<td>GnRH-Th-MAP</td>
</tr>
<tr>
<td>17</td>
<td>GnRH-Th-MAP and oLH</td>
</tr>
<tr>
<td>18</td>
<td>passively immunised with a-GnRH serum</td>
</tr>
<tr>
<td>19</td>
<td>passively immunised with a-GnRH serum and oLH</td>
</tr>
<tr>
<td>20</td>
<td>passively immunised with normal serum (Controls)</td>
</tr>
<tr>
<td>21</td>
<td>PBS (Controls)</td>
</tr>
</tbody>
</table>

B  
**Weights of testes**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weights of testes (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>200 ± 10 p=0.0158</td>
</tr>
<tr>
<td>14</td>
<td>180 ± 10 p=0.0007</td>
</tr>
<tr>
<td>15</td>
<td>150 ± 10 p=0.0066 p=0.0007</td>
</tr>
<tr>
<td>16</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>17</td>
<td>130 ± 10</td>
</tr>
<tr>
<td>18</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>19</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>20</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>21</td>
<td>90 ± 10</td>
</tr>
</tbody>
</table>

Figure 6.13  
Atrophy of the secondary reproductive organs and weights of testes.

A) The urogenital complexes were graded as described in figure 5.15. Mice in groups 14, 16 and 17 all showed complete atrophy of the complex.

B) The testes were weighed and compared to the control group (group 21). The same groups had significantly lighter testes compared to controls. The passively immunised mice also had lighter testes than the controls.
Histological examination of the testes revealed that there were significant differences in the compartmentalisation of the testes in immunised mice compared to controls (figure 6.14 A). The interstitial compartment was reduced in the two immunised groups immunised with GnRH-Th-MAP and GnRH-Th-MAP along with oLH (p=0.0464 and p=0.0424).

6.4.3 RLF expression

RLF expression was down-regulated in one of the immunised groups, the GnRH-Hsp70 immunised mice, p=0.0164 (see figure 6.14 B). The other treated groups did not show a significant change in RLF expression in the testes.
Immunisations with constructs of hCG and oLH and cascade inhibition

Point counts

A) Volume densities of the different compartments of the testis, the cellular, interstitial and lumen compartments. The interstitium was significantly reduced in GnRH-Th-MAP and GnRH-Th-MAP + oLH immunised mice (groups 16 and 17).

B) RLF expression was down-regulated in GnRH-Hsp70 immunised mice (group 14).

Figure 6.14
Point counts and RLF expression in immunised mice
6.5 Discussion

With the aim of affecting the functional levels of LH in male mice they were immunised with either hCG conjugated to Hsp70 or with oLH. The immunised mice responded with a high hCG-specific antibody response in the hCG-Hsp70 immunised mice and with a high oLH-specific antibody response in the oLH immunised mice. In both cases the antibodies cross-reacted with rat LH in ELISA assays, suggesting that they could cross-react with endogenous murine LH as murine LH and rat LH share 98% sequence homology. Ovine LH on its own, i.e. not conjugated to a carrier, resulted in a strong anti-oLH response, although only three of the five oLH immunised mice had rLH cross-reacting antibodies in their sera, it was used in conjunction with GnRH-Hsp70 and GnRH-Th-MAP in an attempt to neutralise both GnRH and LH at once and thereby inhibit two steps of the reproductive hormone cascade. Ovine LH has been used successfully to neutralise endogenous LH in rats (e.g. Wakabayashi and Tamaoki, 1966, Awoniyi et al., 1989). The immunisation schedules have usually entailed repeated administration of oLH to withhold the level of neutralisation and in most cases the use of strong adjuvant, such as Freund's adjuvant.

Immunisation with oLH was also used along with passive immunisations with GnRH-specific rabbit serum. Passive administration of GnRH-specific antibodies should not interfere with the antibody response to oLH and passive immunisation has the benefit of raised GnRH-specific IgG levels immediately after administration, without the lag time of the induction of an immune response to a GnRH construct.

The co-immunisations of GnRH constructs and oLH resulted in high GnRH-specific antibody titres, with little or no difference between the different actively co-immunised groups. When the mice were sacrificed and bled the levels of residual GnRH-specific antibodies in the passively immunised animals were however considerably lower than GnRH-specific antibodies in the actively immunised mice.

Interestingly the oLH-specific antibody titres reached by immunisation with oLH on its own were not reached by immunisations with oLH and GnRH constructs together. Ovine LH-specific antibodies were barely detectable in the group immunised with
Immunisations with constructs of hCG and oLH and cascade inhibition

GnRH-Hsp70 and oLH, and not detectable at all in the group immunised with GnRH-Th-MAP. Only one mouse out of the five passively immunised with anti-GnRH antibodies and actively immunised with oLH showed a detectable oLH-specific antibody response. The lack of response could have been due to epitope competition between the GnRH immunogens and oLH in the actively co-immunised mice. GnRH conjugated to Hsp70 has numerous T cell epitopes and the GnRH-MAP has a T cell epitope incorporated into its structure. These T cell epitopes could compete with the known T cell epitopes on oLH and have higher immunogenicity. The phenomenon of epitope competition does not however explain why oLH did not evoke an LH-specific antibody response in the passively immunised mice. The response to oLH in that case would not have been subjected to the same epitope competition as co-immunisation with a strongly immunogenic construct.

Testosterone levels were reduced in all but two of the immunised groups. This is in contrast to the previous immunisations where the variation within each group was higher and therefore there were less significant differences in the immunised groups compared to the control groups. Testis weight was also reduced in all but three of the immunised groups, the same groups that had reduced testosterone levels, except for the passively immunised and oLH actively immunised group (group 19). The urogenital complex, including the seminiferous vesicles and prostate was atrophied in all the immunised mice, except the group immunised with oLH only. The atrophy was not complete in all of the groups, but the ones with lower testes weights and lower testosterone levels also had the most involuted complexes. The interstitial compartment was only reduced in two of the immunised groups and the RLF expression in the Leydig cells was down-regulated in only one group. The biological effects of the immunisations are summarised in table 6.2.

It is clear that in spite of an LH-specific antibody response in the mice immunised with oLH, the antibodies were not able to completely neutralise endogenous LH and there was no evidence of any biological changes to the reproductive system. Nevertheless, the effect of LH immunisation along with immunisation against GnRH could have had an additive effect. Whether oLH in Ribi adjuvant is not immunogenic enough to make high enough LH-specific antibody titres to neutralise the hormone or whether the
effects of LH immunisation are more pronounced in other species, such as the rat, is not known.

There was, however, clear evidence of a biological effect of the GnRH immunisations. The effect was not enough to disrupt spermatogenesis completely, but the changes in testosterone level, the atrophy of the urogenital complex and the testes, the reduction in interstitium and the down-regulation of the testosterone dependent gene expression of RLF are all indicative of a disruption in the normal reproductive hormone pathway.
<table>
<thead>
<tr>
<th>Group</th>
<th>Immunogen</th>
<th>Antibody titers a-GnRH</th>
<th>Antibody titers a-oLH</th>
<th>Testosterone levels (nmol/l)</th>
<th>Testes weights (mg)</th>
<th>Urogenital complex grades</th>
<th>Point counts reduction in interstitium</th>
<th>RLF expression reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 13</td>
<td>oLH in RIBI</td>
<td>NA</td>
<td>6640</td>
<td>42.92</td>
<td>210</td>
<td>3.0</td>
<td>12.8/121 (not significant)</td>
<td>0.4893 (not significant)</td>
</tr>
<tr>
<td>Group 14</td>
<td>GnRH-Hsp70 in RIBI</td>
<td>1640</td>
<td>NA</td>
<td>0.16</td>
<td>180</td>
<td>1.0</td>
<td>8.8/121 (not significant)</td>
<td>0.2184 (p=0.016)</td>
</tr>
<tr>
<td>Group 15</td>
<td>GnRH-Hsp70 + oLH in RIBI</td>
<td>1110</td>
<td>140</td>
<td>22.23</td>
<td>210</td>
<td>2.0</td>
<td>12.8/121 (not significant)</td>
<td>0.4386 (not significant)</td>
</tr>
<tr>
<td>Group 16</td>
<td>GnRH-Th-MAP in RIBI</td>
<td>1100</td>
<td>NA</td>
<td>1.34</td>
<td>180</td>
<td>1.0</td>
<td>6.4/121 (p=0.0464)</td>
<td>0.6171 (not significant)</td>
</tr>
<tr>
<td>Group 17</td>
<td>GnRH-Th-MAP + oLH in RIBI</td>
<td>1240</td>
<td>not detectable</td>
<td>0.16</td>
<td>180</td>
<td>1.0</td>
<td>7.0/121 (p=0.424)</td>
<td>0.504 (not significant)</td>
</tr>
<tr>
<td>Group 18</td>
<td>Passive imm. a-GnRH</td>
<td>480</td>
<td>NA</td>
<td>1.03</td>
<td>180</td>
<td>2.0</td>
<td>9.3/121 (not significant)</td>
<td>0.8267 (not significant)</td>
</tr>
<tr>
<td>Group 19</td>
<td>Passive imm. a-GnRH + oLH in RIBI</td>
<td>350</td>
<td>51</td>
<td>3.58</td>
<td>180</td>
<td>2.0</td>
<td>12.6/121 (not significant)</td>
<td>0.8310 (not significant)</td>
</tr>
<tr>
<td>Group 20</td>
<td>Normal rabbit serum</td>
<td>NA</td>
<td>NA</td>
<td>20.90</td>
<td>220</td>
<td>2.4</td>
<td>10.2/121 (NA)</td>
<td>NA</td>
</tr>
<tr>
<td>Group 21</td>
<td>PBS in RIBI</td>
<td>NA</td>
<td>NA</td>
<td>38.37</td>
<td>210</td>
<td>3.0</td>
<td>12.0/121 (NA)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 6.2
A summary of the results of GnRH and LH immunisations of mice

Significant differences between immunised mice and control mice (p<0.05) are coloured yellow.
Chapter 7  General Discussion
General Discussion

7.1 Aims of the project

The aim of the work presented in this thesis was to develop an improved GnRH-based immunosterilisation vaccine and to test the hypothesis that a vaccine targeting two hormones of the reproductive hormone pathway simultaneously is more efficient than a vaccine against single stages of the pathway. To evaluate the effect on the reproductive system and the degree of neutralisation of the hormones the effect of immunisation on spermatogenesis, serum testosterone levels and testosterone dependent gene expression in the testes was analysed.

The results of the experiments presented here however do not indicate an additive effect of immunising against two consecutive hormones in the pathway. Constructs of GnRH or GnRH analogues attached to carrier molecules, T cell epitopes or expressed on the surface of bacteriophage particles, were used as immunogens in male mice along with oLH. The GnRH constructs all elicited antibody responses to GnRH and had to various extents deleterious effects on the reproductive system. Ovine LH on its own also elicited LH-specific antibody responses but oLH given along with a GnRH construct did not have an enhanced effect on the reproductive system, as was expected, and the doubly immunised animals did not mount a detectable LH-specific antibody response.

7.2 Immunogenicity of the constructs

All of the animals immunised with constructs of GnRH developed GnRH-specific antibody responses (except one animal immunised with GnRH-TT), to varying degrees though. The highest antibody titres were found in mice immunised with GnRH conjugated to Hsp70, whether administered with or without an added adjuvant. The
hormone-specific antibody response to some extent is dependent on the immunogenicity of each carrier molecule, the number of T cell epitopes supplied by the carrier and their accessibility in the construct. Tetanus toxoid has been used as a carrier in numerous immunisation experiments (e.g. Ladd et al., 1990, Ladd et al., 1994) with variable responses. Goubau and co-workers found that GnRH coupled to equine serum albumin (ESA) and OVA were more effective in raising anti-GnRH antibody titres in sheep than GnRH coupled to TT and KLH (Goubau et al., 1989). They proposed that the variability in antibody responses to GnRH conjugated to TT could be due to species variability and also to the variable degree of purification of different preparations of TT. The carrier proteins TT, KLH and Hsp70 were conjugated to GnRH at various different molar ratios to optimise the coupling procedure to produce immunogens with the maximum number of GnRH molecules per carrier.

To be acceptable as an immunogen for commercial use in animals for food production or pets, or for human use against androgen-dependent carcinomas, the vaccine construct used must have a uniform molecular structure within and between batches. Most GnRH vaccines to date depend on the chemical coupling of GnRH to a carrier protein, which is time consuming and variable and does not ensure that the produced immunogen is homogenous. Alternatives include bacterial expression of GnRH constructs using recombinant DNA technology. Quesnell and colleges found that two fusion proteins incorporating seven copies of GnRH with OVA or thioredoxin, respectively, expressed in E. coli, were effective in inducing GnRH-specific antibodies and reducing testicular and accessory sex gland weights and function but were not 100% effective in all the immunised animals (Quesnell et al., 2000). GnRH displayed on the surface of bacteriophage particles has the advantage over chemical coupling procedures that the bacteriophages can be grown and extracted for use as immunogens in large quantities under standardised conditions. The bacteriophage particles themselves are highly immunogenic and provide the necessary T cell epitopes to activate the humoral immune response against the fused peptide (Greenwood et al., 1991).

In an attempt to compensate for the variability in response of animals to GnRH immunisations constructs have been made with a number of tandem GnRH repeats
conjugated to a carrier molecule. The GnRH-MAPs used in this project are branched constructs containing more than one GnRH peptide. They were synthesised with either a T helper cell epitope in tandem with the GnRH peptide connected to the lysine core or without it, but then administering it along with the MAP in the adjuvant mixture. The MAP construct is practical as it is easily reproduced by peptide synthesis, without the variation mentioned above, but the mass production of such a construct is expensive and time consuming. Synthesis using recombinant DNA procedures could possibly overcome these shortcomings.

7.3 Biological effects of the immunisations

If the pituitary gland is removed (hypophysectomy) the testes shrink, spermatogenesis is arrested, the Leydig cells become involuted, serum testosterone levels fall and testosterone-dependent accessory sex glands hypotrophy. Ghosh and colleagues found that hypophysectomy had an immediate effect on germ cells, width and length of the seminiferous tubules, testes' weights and volume of the interstitium and that the Leydig cells and their subcellular organs were significantly reduced. Sertoli cells, however, did not show the same effect until after long-term hypophysectomy (Russell et al., 1992, Ghosh et al., 1992). If GnRH is successfully neutralised by GnRH-specific antibodies the effects on the reproductive system in a male animal should reflect the effects of a hypophysectomy.

The effects seen in the experiments presented here were drastic on the accessory reproductive organs, such as the seminiferous vesicles and the prostate. The testes were reduced in weight in some of the treatment groups compared to control animals. Testosterone levels were significantly reduced in 6 out of 13 treated groups. In all the groups there was great variation in serum testosterone levels, which correlated with GnRH-specific antibody levels (Pearson's correlation coefficient = -0.238; p<0.05 for n=84). The more dramatic reduction in size of the seminiferous vesicles and prostate gland than in the testes is possibly due to a higher proportion of androgen-dependent cells in the seminiferous vesicles and the prostate compared to LH-dependent cells in the testes.
More subtle changes could be seen by looking at the histology and composition of the different compartments of the testes. The different compartments vary in sensitivity to reduced testosterone levels. Testosterone dependent gene expression in the testes can also be indicative of reduced levels of available testosterone in the testes even though the serum levels are found to be unaffected.

Complete testosterone withdrawal by depletion of Leydig cells leads to a 50% reduction in interstitial fluid in the testes and a reduction in the seminiferous tubule fluid following a decrease in protein secretion by the seminiferous tubules (Sharpe et al., 1994). Similar effects were found after immunoneutralisation of LH (Sharpe et al., 1992). Seminiferous tubule fluid reduction leads to a stage-dependent reduction in lumen size. Blood flow and vasomotion in the testis is controlled by testosterone (Collin et al., 1993, Damber et al., 1992). As the interstitial fluid is formed by vasomotion any reduction in testosterone has an effect on interstitial fluid production and the volume of the interstitium.

In the GnRH-Hsp70 and GnRH-MAP immunised mice the volume of the interstitial compartment of the testes was significantly reduced. The same reduction was not found for the oLH immunised mice, but animals immunised with both GnRH and oLH showed a reduction. Testosterone dependent RLF gene expression was reduced in the same GnRH immunised groups but oLH immunisation did not have an effect on the expression, neither did co-immunisation of oLH and GnRH constructs. Immunisation with oLH did not have an additive effect on any of the biological markers mentioned.

In mice immunised with GnRH-Hsp70 round spermatids in the meiotic stages of the spermatogenic cycle were observed released into the seminiferous vesicle lumens. Testosterone withdrawal has been shown to induce the detachment of round spermatids at stages VII-VIII of the spermatogenic cycle from the seminiferous epithelium in rats. Around stage VII-VIII, which have been shown to be particularly sensitive to testosterone withdrawal, a tight association between the spermatid and Sertoli cell develops. Testosterone possibly takes part in the regulation of this connection via cell adhesion molecules (O'Donnell et al., 1996).

It is of interest that the GnRH-immunised 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 an antigen.

The biological effects of immunisations with GnRH constructs and oLH are summarised in table 7.1.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Immunogen</th>
<th>Antibody titre anti-GnRH</th>
<th>Antibody titre anti-oLH</th>
<th>Testosterone (nmol/l)</th>
<th>Testes' weights (mg)</th>
<th>Urogenital complex</th>
<th>Point counts reduction in interstitium</th>
<th>RLF expression down regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>GnRH-TT in RIBI</td>
<td>intermediate</td>
<td>NA</td>
<td>23.10</td>
<td>224</td>
<td>not analysed</td>
<td>not analysed</td>
<td>not analysed</td>
</tr>
<tr>
<td>4</td>
<td>Phage display of GnRH in RIBI</td>
<td>intermediate</td>
<td>NA</td>
<td>32.15</td>
<td>232</td>
<td>not analysed</td>
<td>not analysed</td>
<td>not analysed</td>
</tr>
<tr>
<td>7</td>
<td>GnRH-Hsp70 in ICF</td>
<td>high</td>
<td>NA</td>
<td>6.26</td>
<td>290</td>
<td>not affected</td>
<td>not affected</td>
<td>reduced (p&lt;0.05)</td>
</tr>
<tr>
<td>8,14</td>
<td>GnRH-Hsp70 in RIBI</td>
<td>high</td>
<td>NA</td>
<td>1.32</td>
<td>195</td>
<td>atrophied</td>
<td>reduced (p&lt;0.05)</td>
<td>reduced (p&lt;0.05)</td>
</tr>
<tr>
<td>15</td>
<td>GnRH-Hsp70 + oLH in RIBI</td>
<td>high</td>
<td>intermediate</td>
<td>22.23</td>
<td>213</td>
<td>atrophied</td>
<td>not affected</td>
<td>reduced (p&lt;0.05)</td>
</tr>
<tr>
<td>1,11</td>
<td>GnRH-MAP + Th in RIBI</td>
<td>intermediate</td>
<td>NA</td>
<td>2.73</td>
<td>214</td>
<td>not affected</td>
<td>reduced (p&lt;0.05)</td>
<td>not affected</td>
</tr>
<tr>
<td>10,16</td>
<td>GnRH+Th-MAP in RIBI</td>
<td>high</td>
<td>NA</td>
<td>3.65</td>
<td>190</td>
<td>atrophied</td>
<td>reduced (p&lt;0.05)</td>
<td>reduced (p&lt;0.05)</td>
</tr>
<tr>
<td>17</td>
<td>GnRH-Th-MAP + oLH in RIBI</td>
<td>high</td>
<td>not detectable</td>
<td>0.16</td>
<td>178</td>
<td>atrophied</td>
<td>reduced (p&lt;0.05)</td>
<td>not affected</td>
</tr>
<tr>
<td>18</td>
<td>Passive immunisation with anti-GnRH</td>
<td>intermediate</td>
<td>NA</td>
<td>1.03</td>
<td>176</td>
<td>atrophied</td>
<td>not affected</td>
<td>not affected</td>
</tr>
<tr>
<td>19</td>
<td>Passive immunisation with anti-GnRH + oLH in RIBI</td>
<td>intermediate</td>
<td>not detectable</td>
<td>3.58</td>
<td>182</td>
<td>atrophied</td>
<td>not affected</td>
<td>not affected</td>
</tr>
<tr>
<td>13</td>
<td>oLH in RIBI</td>
<td>NA</td>
<td>high</td>
<td>42.92</td>
<td>212</td>
<td>not affected</td>
<td>not affected</td>
<td>not affected</td>
</tr>
</tbody>
</table>

Table 7.1 Summary of the biological effects of immunisations with GnRH constructs and oLH (pooled data)

The values in blue differ significantly from controls (p<0.05)
NA = Not applicable
7.4 Cascade inhibition

When oLH was administered along with either GnRH-Hsp70 or GnRH-MAP the immune response mounted was exclusively against GnRH in the group immunised with GnRH-MAP and oLH but an intermediate oLH-specific antibody response was raised in animals immunised with GnRH-Hsp70 and oLH. Ovine LH on the other hand elicited a strong oLH-specific antibody response. The GnRH constructs exhibited dominance of the immune response. For this phenomenon there are numerous possible explanations.

The carrier molecule Hsp70 has numerous $T_H$ cell epitopes (Oftung et al., 1994, Adams et al., 1994), which accounts for its strong immunogenicity when used as a carrier molecule. In addition it has active adjuvant properties (Barrios et al., 1992, Lehner et al., 2000, Lussow et al., 1991, Suzue and Young, 1996). There are many more copies of the same hapten molecule (GnRH peptide) coupled to Hsp70 molecule than there are copies of each B cell epitope on the oLH molecule. In the case of GnRH-MAP there are multiple copies of GnRH molecule (the B cell epitope) and $T_H$ epitope peptide per molecule of construct (eight of each), but on oLH there is only one copy of each epitope per molecule. Different epitopes on the same antigen molecule can compete for the immune response. A shift in epitope dominance is clearly seen in mutation experiments on hCG, where the immune dominance changes after mutation of certain epitopes (Porakishvili et al., 1998, Chiesa et al., 2001).

Immunoneutralisation of GnRH occurs when antibodies of adequate affinity bind tightly to the hormone and render it biologically ineffective. The site of immunoneutralisation of GnRH is most likely in the hypothalamic portal vessels. In the present study the affinity of GnRH-specific antibodies was not measured. In previous work however analysis of antibody affinity showed great variation between individual animals (e.g Quesnell et al., 2000). Mice who had low GnRH-specific antibody titres but a good biological response had high affinity antibodies and mice who had high antibody titres and no biological response had antibodies of low affinity. The age that a neutralising antibody response to GnRH appears in the animal is critical.
for the outcome of the immunisation. When the animals are bled and antibody titres evaluated it is hard to predict at what time point the titres reached high enough levels necessary to neutralise the hormone.

There are therefore numerous aspects that need to be considered when formulating an immunosterilisation vaccine. The antibody titre of an antisera is not the determining factor for immunoneutralisation to succeed, the affinity needs to be sufficiently high and the immune response mounted against epitopes that when bound to antibody render the hormone biologically inactive.

Summary and conclusion

Of the GnRH-based immunosterilisation vaccine candidates tested here, the GnRH-Hsp70 and GnRH-Th-MAP constructs proved to be highly immunogenic, resulting in high GnRH-specific antibody titres, and effective in inducing atrophy of secondary sex organs, histological changes in the testes and reducing testes’ weights. The vaccine formulations used are improvements on previous immunosterilisation vaccines as they required only two boosts of immunogen and were administered in a mild adjuvant. GnRH-Hsp70 even showed comparable immunogenicity when administered without active adjuvant in ICF. These formulations are therefore strong candidates for further research into an effective, safe and practical immunosterilisation vaccine.

Immunisation with oLH resulted in high oLH-specific antibody titres. The additive effects of GnRH and LH co-immunisations were however not detectable in the biological effects on the reproductive system and oLH-specific antibody titres were in fact negligible in the co-immunised groups. The reasons for such non-responsiveness to oLH include possible epitope dominance and suppression of the immune response to the less immunogenic construct. Further research is needed to find ways to overcome the epitope dominance and to make a vaccine incorporating both GnRH and LH enhancing the effect of immunisations against GnRH on its own.
7.5 Further work on immunosterilisation vaccines

Modern vaccine development is greatly focused on antigens produced by synthetic chemistry and by genetic engineering techniques. These methods can potentially provide safer and more efficient antigens that can be produced in large quantities. A drawback to these often simplified and structurally well defined antigens is that recombinant or synthetic antigens are often not sufficiently immunogenic to produce a strong and efficient immune response on their own. They require either an adjuvant to stimulate the immune response to the antigen and/or they need to be conjugated to carrier molecules with intrinsic adjuvanticity such as the Hsp70 carrier used here. However, the use of adjuvants to enhance the immune response is limited by the lack of adjuvants suitable for use in humans and livestock.

Currently aluminium compounds are widely used in veterinary practice and are the only acceptable adjuvants used in man. Other adjuvant formulations, including the Ribi adjuvant system used here, have shown little toxicity and have reached the stage of clinical trials in humans (reviewed in Baldridge and Crane, 1999, Ulrich and Myers, 1995) and could be used routinely in the near future in livestock as well as in humans.

The development of an immunosterilisation vaccine for commercial use also needs to take into account the number of immunisations needed to reach the threshold levels of neutralising antibody to affect fertility. The use of a GnRH vaccine is hampered by the necessity of frequent immunisations and the lag period in antibody production after the initial immunisation, delaying the onset of neutralisation of the hormone. Controlled release of a GnRH vaccine administered in biodegradable microspheres could overcome the need for multiple immunisations. Immunisations of rats with such a vaccine resulted in less delay of the build up of antibody titres and the levels of antibody also remained higher for longer (Diwan et al., 1998).

Although advances have been made in terms of efficacy and safety, present immunosterilisation vaccine formulations are still not ideal for routine use in domestic animals and livestock. As stated before there may be a number of confounding factors responsible for the apparent lack of effect of the cascade inhibition immunisations in
the current work. However, further studies using different carriers or adjuvant systems may confirm the additive affect of neutralising two consecutive steps of a hormone cascade. Safer and more effective adjuvants are also being developed and methods such as controlled release are likely to produce a more cost-effective technique.

Further advances in the development of immunosterilisation vaccines will undoubtedly lead to more humane treatment of animals and improved veterinary procedures. An immunosterilisation vaccine is unlikely to be used in fertility control for humans but immunisation against GnRH and the use of GnRH agonists and antagonists is of interest in steroid-dependent pathologies such as prostate cancer and some have reached the stage of advanced clinical trials.
Appendices
Appendix A – Sequences

Cloning of GnRH into the phagemid pC89

Sense primer (GnRH-Phage-F):
5' aaaaagcggcctttgactccctgc 3'

Antisense primer (GnRH-Phage-R):
5' gcgggatccctccagggcgctttgactccctgc 3'

The BamHI restriction site is in bold and the GnRH antisense sequence is underlined.

GnRH

Human GnRH:
CAG CAC TGG TCC TAT GGA CYG CGC CCT GGA

Gln his trp ser tyr gly leu arg pro gly

LH

Ovine alpha-LH

CTGCCACAA CACATCCTTC CAAGATCCAG AGTTTGCAGG AGAGCTATGG
ATTGCTACAG AAGATATCGG GCTGTCATTTC TGGTCATGCT GTCCATGGTC
CTGCATATTC TCTGATGGGAGAGCTATGG
TCCAGAATGT AAACTAAAGG AAAACAAATA CTTCTCCAAG CTGGGTGCC
CCATCTATCA GTGTATGGGC TGTTG CTTCT CCAGGGCATA CCCGACTCCC
GCAAGGTCCA AGAAGACAAT GTTGGTTCCA AAGAATATTA CCTCGGAGGC
CACGTCGCTGT GTGGCCAAAT CATTTACTAA GGCCACAGTG ATGGGAAACG
Appendices

CCAGAGTGGA GAACCACACG GACTGCCACT GTAGCACTTG TTACTACCAC
AAGTCGTAGC TTCCATGTGT GCCAAGGGCT GCGCTGACGA CTGCTGACCC
GTGCGATGGC ACTGAGTGTC GCCACCTCCT CTTACCAGA TTTCTGACAC
GCTTCAGTCA TACACTGCTG CTTTCCTGTC ACATCCCTTA TACTTCAGTA
CCATCGACAG TCTCTTCTCA TTAGGGGAAA ATGTTATCT ACCATGGTCC
CATCAGAAT

Ovine beta-LH:

GCTCCAGGGG CTGCTGCTGT GGCTGCTGCT GAGCCCAAGT GTGGTGTGGG
OGTCCAGGGG CCCCTTCGG CCACCTGTC GCCTGCCTAA CGCAACCTC
GCTGCAGAGA ATGAGTTCTG CCCAGTCTGC ATCACCTTCA CCACCAGCAT
CTGTGCCGGC TACCTGCTTA GCATGGTTCG AGTACTGCA GCTGCTGCTTC
CTCCCGTGGC TCAGCCAGTG TGCACTTACC GTGAGCTGCG CTGCCCTCTC
GTCCGCCTCC CTGCTGGCCC ACCTGGTGA GACCCCATAG TCTCCTCCC
TGTCGGCCCTC AGCTGCGCCT GGGGCCCTG CGTCTCAGT AGCTGTGACT
GTGGGGGCTC CAGGACTCAA CCAATGACCT GTGACCTCC CCACCTCCCC
GGCCTTCTCC TCTCTGATG CCCACCCCAT AACTCCCCAT TCTTCTGGAG
CCAGCAGGTC TTCACCACCT CTCCCAATAA AGGCTTACAA ACTGC
Appendix B

Buffer I (for competent cells)
10 mM MES
100mM RbCl
10 mM CaCl$_2$
50 mM MnCl$_2$
pH adjusted to pH 5.8 with addition of KOH

Buffer II (for competent cells)
10 mM Pipes (pH 6.5)
75 mM CaCl$_2$
10 mM RbCl
15% (v/v) glycerol

Solution I / GTE (for DNA preps)
50 mM glucose
10 mM EDTA
25mM Tris pH 8.0

Solution II (for DNA preps)
0.2 M NaOH
1% SDS

Solution III (for DNA preps)
5 M potassium acetate pH 4.8

Sodium citrate (for RNA extraction)
0.75 M Sodium citrate
DEPC treated

10% sarkosyl (w/v) (for RNA extraction)
10% Sarkosyl (w/v)
DEPC treated

Solution D (for RNA extraction)
4.2 M Guanidinium isothiocyanate
1.3 mM Sodium citrate
10% Sarkosyl (w/v)
5 mM 2-Mercaptomethanol (7.2 µl/ml)
ddH$_2$O

2 M Sodium acetate (pH 4.1) (for RNA extraction)
2 M Sodium acetate
ddH$_2$O
pH adjusted to 4.1 with acetic acid
DEPC treat

3 M Sodium acetate (pH 5.6) (for RNA extraction)
3 M Sodium acetate
ddH₂O
pH adjusted to 5.6 with acetic acid
DEPC treated

Chloroform/isoamylalcohol (24:1) (for RNA extraction)
48 ml Chloroform
2 ml Isoamylalcohol

20 x MEA
200 mM MOP
10 mM EDTA
1 M Sodium acetate (anhydrous)
ddH₂O
pH adjusted to 7.2 using NaOH

20 x SSC
3M NaCl
300 mM Na₃C₆H₅O
pH adjusted to 7.4

RNA loading dye
20 µl 20 x MEA
140 µl Formaldehyde (35%)
400 µl Formamide
40 µl Bromophenol blue and xylene cyanol
Add 1 µl of ethidium bromide (10 mg/ml) per 100 µl of loading buffer

50 x TAE buffer
1.0 M Tris base
6% (v/v) Glacial acetic acid
0.5 M EDTA (pH 8.0)
ddH₂O

10 x TBE buffer
1.8 M Tris base
1.8 M Boric acid
0.5 M EDTA (pH 8.0)
ddH₂O

SDS stacking gel
ddH₂O
18% Bisacrylamide stock
0.13 M Tris (pH 6.8)
0.2% SDS
0.2% APS
Temed
**Appendices**

**SDS 20% resolving gel**
- ddH₂O
- 20% Bisacrylamide stock
- 0.23 M Tris (pH 8.8)
- 0.25% SDS
- 0.25% APS
- Temed

**Running buffer for westerns (5x)**
- 25 mM Tris
- 250 mM glycine (pH 8.3)
- 0.1% SDS
- ddH₂O

**2 x SDS gel loading buffer**
- 100 mM TrisHCl (pH 6.8)
- 200 mM dithiothreitol (or without DTT)
- 4% SDS
- 0.2% bromophenol blue
- 20% glycerol

**DNA loading buffer (6x)**
- 1.2 M Sucrose
- 0.4 M Bromophenol blue
- 60% TrisHCl (pH 8.0)
- 1mM EDTA
- TE buffer (pH 8.0)

**EDC conjugation buffer (for EDC conjugation reactions)**
- 0.1 M MES
- 0.9 M NaCl
- 0.02 % NaN₃
- ddH₂O

**GDA conjugation buffer (for GDA conjugation reactions) – phosphate buffer (pH 5.0)**
- 0.11 M NaH₂PO₄·2H₂O
- pH adjusted to pH 5.0 with NaOH

**LB medium**
- 1.0% (w/v) Bacto-tryptone
- 0.5% (w/v) Bacto-yeast extract
- 0.2 M NaCl
- ddH₂O

**2xTY medium**
- 1.6% (w/v) Bacto-tryptone
- 1.0% (w/v) Bacto-yeast extract
- 0.1 M NaCl
- ddH₂O
**Agar plates**
Agar added to medium (either LB or 2xYT), 15 g/l and autoclaved
Approximate volume of agar-medium on each plate is 15 ml

**Top agar**
Agar added to medium (either LB or 2xYT), 7 g/l and autoclaved
Approximate volume of top agar poured onto each plate is 3 ml

**10 x PBS**
1.4 M NaCl
30 mM KCl
100 mM Na₂HPO₄
18 mM KH₂PO₄
ddH₂O
pH adjusted to 7.4 with HCl

**PBS/Tween**
1 x PBS
0.1 % Tween 80

**10% SDS**
10% (w/v) SDS (electrophoresis-grade)
ddH₂O
heated to 68°C to dissolve and pH adjusted to 7.2 with HCl

**Tissue culture buffers**

**Na₂HPO₄**
i) 70 mM Na₂HPO₄ pH 10.0
ii) 70 mM NaH₂PO₄ pH 3.5
i) and ii) mixed until pH reaches 7.1

**Hepes x 2**
0.042 M Hepes
0.27 M NaCl
ddH₂O
pH adjusted to 7.1 with NaOH

**Hepes-PO₄**
9.8 ml Hepes x 2
0.2 ml Na₂HPO₄

**ELISA buffers**

**Buffer A:**
0.2 M anhydrous sodium carbonate

**Buffer B:**
0.2 M sodium bicarbonate
CBB (carbonate-bicarbonate buffer)
For pH 9.6 16.0 ml of buffer A and 34.0 ml of buffer B were mixed in 200 ml of ddH$_2$O
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