Immunization of bull calves with a GnRH analogue–human serum albumin conjugate: effect of conjugate dose, type of adjuvant and booster interval on immune, endocrine, testicular and growth responses

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Bull calves were immunized with GnRH analogue–human serum albumin (HSA-Cys-Gly-GnRH) conjugate to determine the effects of dose, adjuvant type and interval between primary and booster injections on plasma testosterone and LH concentrations, and testes and body growth. Friesian bull calves aged between 8 and 10 weeks (n = 72) were blocked according to age and weight and, within block, randomly assigned to 12 treatment combinations (n = 6 per treatment combination) in a 3 × 2 × 2 factorial plan. Main effects were (i) conjugate dose (0.0, 0.1 or 1.0 mg HSA-Cys-Gly-GnRH), (ii) adjuvant (diethylaminoethyl-dextran or non-ulcerative Freund’s adjuvant), and (iii) interval between primary (day 0) injection and booster injection (day 28 or 56). Plasma testosterone and LH concentrations and antibody titres were determined in blood samples collected at 14 day intervals during the experiment (140 days). Testicular measurements were taken in situ every 28 days. Antibody titres (% binding at 1:160 dilution) were ≥10% 28 days after booster injection and remained high for 140 days in 47 of 48 GnRH-immunized bulls. The mean titre was higher (P < 0.05) in response to the 1.0 mg dose compared with the 0.1 mg dose (37.7% versus 29.6% binding, respectively; pooled SED 2.55%). Mean LH and testosterone concentrations were reduced (P < 0.05) in immunized animals compared with controls. However, the 1.0 mg dose decreased mean testosterone concentrations by a greater extent (P < 0.001) than either the 0.1 mg or 0.0 mg doses. Testes length and depth, and scrotal circumference were decreased (P < 0.001) in immunized animals compared with controls; however, the 1.0 mg dose decreased (P < 0.001) testes parameters to the greatest extent. There was no effect of conjugate dose on average daily gain in body mass. It is concluded that (i) dose of conjugate, type of adjuvant and interval between primary and booster injections affected antibody titres, (ii) the use of 0.1 or 1.0 mg of HSA-Cys-Gly-GnRH decreased LH and testosterone concentrations, and testicular development throughout the experiment, without adversely affecting body growth, and (iii) an effective protocol is 1.0 mg GnRH–HSA conjugate, given in the adjuvant diethylaminoethyl dextran, with a primary–booster interval of 56 days.

Introduction

Testicular development, onset of puberty and semen production are regulated by a complex interplay of hypothalamic, pituitary and testicular hormones. Rodriguez and Wise (1989) demonstrated that there is an age-associated increase in the pulsatile release of GnRH from the hypothalamus in the prepubertal bull, which results in an increased pulsatile release of LH from the anterior pituitary. This increase in pulsatile LH secretion initiates the onset of puberty in bulls (Amann and Walker, 1983; Amann et al., 1986; Wise et al., 1987) by increasing serum testosterone concentrations (McCarthy et al., 1979). Testosterone induces aggressive and sexual behaviour in males (Johnson and Whalen, 1989; Albert et al., 1990), either directly or via the action of oestriadiol that is derived by aromatization of testosterone in upper brain centres (Balthazart, 1990). Thus, GnRH is a key hormone regulating the...
hypothalamic–pituitary–testicular axis and, consequently, aggressive and sexual behaviour in bulls.

Accordingly, a practical research goal is to neutralize GnRH activity and thus reduce testosterone to concentrations at which sexual and aggressive behaviour are reduced but at which the anabolic advantages of the normal male are maintained. Some workers have approached this goal in bulls by administering GnRH in high doses to downregulate GnRH receptors (Melson et al., 1986; Rechenberg et al., 1986; Ronayne et al., 1993). However, these studies have demonstrated that administration of GnRH or a GnRH analogue for short or long periods ultimately increased rather than decreased testosterone concentrations.

An alternative approach is to immunoneutralize GnRH by active immunization against a GnRH–protein conjugate in a suitable adjuvant. Previous studies with native GnRH and GnRH analogues have been reviewed by several authors (Falvo et al., 1985; Fraser, 1986; Silversides et al., 1988a, b; Gonzalez et al., 1990). Studies in male cattle (Robertson et al., 1979, 1981, 1982, 1984; Jeffcoate et al., 1982; Lobley et al., 1992) generally show that a percentage of animals that develop significant GnRH antibody titres express a temporary castration effect lasting 6–9 months that is manifested by low testosterone concentrations, involution of the testes and azoospermia. However, even with repeated booster immunizations in some studies (Robertson et al., 1979, 1981, 1982, 1984; Gonzalez et al., 1990), only up to 50% of the GnRH-immunized animals develop significant antibody titres and have periods of reduced testosterone concentrations and testicular size. Although immunization against GnRH has proved to be partially effective, there are problems relating to consistency and longevity of the immune response both within and between animals in most studies in the literature.

An optimum dose of GnRH conjugate has not been determined in farm animals; however, doses between 0.05 and 5.00 mg GnRH conjugated to various protein carriers have been shown to elicit immune responses with varying efficiency (Schanbacher, 1984a; Falvo et al., 1986; Goubau et al., 1989b; Lobley et al., 1992; Adams and Adams, 1992). Carson et al. (1992) reported that 1.0 mg of a GnRH conjugate given to pubertal bulls (n = 2) was more effective than 4.0 mg. An optimum primary–booster interval has not been determined in any of the studies carried out to date. In addition, a suitable effective alternative to the oil-based adjuvants (e.g. Freund’s complete adjuvant and non-sterilized Freund’s adjuvant (NUFA)) has not been reported. The more successful studies in bulls have included Freund’s complete adjuvant in the immunogen (Jeffcoate et al., 1982; Adams and Adams, 1992). However, oil-based adjuvants are not an option in the development of a practical GnRH immunization regimen, owing to poor degradation of the mineral oil base (Langer, 1981) and local irritation at the site of injection that results in granulomas and abscessation (Chapel and August, 1976; Goubau et al., 1989b).

Thus, part of the problem is to achieve consistently high antibody titres in all treated animals using a safe adjuvant, and an optimum conjugate dose and primary–booster interval. The aim of this experiment was therefore to carry out a dose-titration study of GnRH conjugated to human serum albumin (HSA-Cys-Gly-GnRH) using two different adjuvants and two different booster intervals, to select an effective immunization protocol for all immunized bulls and, consequently, to determine the effect of treatment on plasma LH and testosterone concentrations and on testes and body growth.

Materials and Methods

Animals and treatments

Seventy-two 8–10-week-old Friesian bull calves (mean body mass of 70 ± 0.7 kg) were blocked (n = 6) according to age and mass and, within block, randomly assigned to 12 treatment combinations (n = 6 per treatment combination) in a 3 × 2 × 2 factorial plan. The main effects were as follows.

(i) Dose: 0.0, 0.1 or 1.0 mg HSA-Cys-Gly-GnRH.

(ii) Adjuvant: diethylaminomethyl-dextran (DEAE-D: Sigma Chemical Co, Poole) or NUFA (Guildhay Antisera, University of Surrey, Guildford).

(iii) Interval between primary (day 0) and booster (day 28 or 56) injections.

Animals were housed together on slats throughout the experiment (140 days) and fed silage ad libitum and 2 kg barley per animal day⁻¹.

Preparation of conjugate

The conjugate was prepared by coupling a cysteine-containing analogue of GnRH via a thioether link to HSA that had been previously modified with maleimido groups. The experimental procedure was a modification of that reported by Morrison et al. (1987). The GnRH analogue used was Cys-Gly-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ (Cys-Gly-GnRH). It was synthesized on methylenebendhydramine resin using an ACT (Advanced Chemical Technology) series 200 automated peptide synthesizer (Louisville, KY). The peptide was cleaved from the resin with hydrogen fluoride—anisole and purified by gel filtration on Sephadex G-25 (Pharmacia, Piscataway) followed by preparative HPLC on a 1.5 cm × 45 cm column of silica (10–15 µm, Vydac C18, Separations Group, CA). Peptide purity was assessed by analytical HPLC, TLC and amino acid analysis of acid hydrolysates. Synthesis was performed in the Department of Medicine, Tulane University Medical Center, New Orleans, LA.

HSA (Sigma Chemical Co) was dissolved in 0.5 ml 0.2 mol sodium borate 1⁻¹, 0.15 mol NaCl 1⁻¹ (pH 8.3) and reacted with a 20 molar excess of N-γ-maleimidobutryl oxyxysuccinimide (GMBS, Calbiochem, Lucerne) dissolved in 20 µl dry freshly distilled N, N' dimethylformamide (Pierce, Rockford, IL). The reaction was allowed to proceed for 5 min at 23°C, after which the mixture was centrifuged at 5000 g for 5 min in an Eppendorf centrifuge and applied to a column (0.9 cm × 30 cm) of Sephadex G-25 pre-equilibrated in degassed 0.1 mol Hepes 1⁻¹, 0.15 mol NaCl 1⁻¹ (pH 7.0). Elution was monitored by UV absorption and the material appearing in the void volume was transferred to a stoppered vial: Cys-Gly-GnRH (dissolved in degassed 0.1 mol Hepes 1⁻¹, 0.1 mol NaCl 1⁻¹, pH 7.0) was added dropwise with stirring to a 10 molar excess. This mixture was stirred at 23°C for 6 h and then overnight at 4°C. The extent of protein substitution was
estimated by back-titrating the available free thiol in the mixture at regular intervals using 5, 5′-dithiobis (2-nitrobenzoic acid) (Sigma Chemical Co) according to the method of Deakin et al. (1963). Alternatively, the conjugate was subjected to SDS gel electrophoresis on Phastgel (Pharmacia) and the increase in molecular weight determined. Typically, 6–8 moles of Cys-Gly-GnRH were coupled per mole of HSA. After conjugation, the mixture was exhaustively dialysed against distilled water, lyophilized and stored at 4°C in a desiccator until required for immunization.

**Immunization protocol**

The animals assigned to receive 0.0 mg HSA-Cys-Gly-GnRH (n = 24) were given the appropriate dose of HSA at the scheduled times. The immunogens were prepared as follows.

**Conjugate in DEAE-D.** DEAE-D (0.1 g ml\(^{-1}\)) was dissolved over low heat in sterile distilled water and the pH adjusted to between 7.3 and 7.5 using saturated Tris (Sigma Chemical Co) solution. The Cys-Gly-GnRH-HSA was dissolved in a sterile saline solution (0.9%, pH 7.2) and this was mixed with the DEAE-D solution for 2 h before administration at a final volume of 5 ml per animal.

**Conjugate in NUFA.** Corynebacterium parvum (C. parvum; Coparvax\(^{10}\), Calmic Medical Division, The Wellcome Foundation Ltd, London) was incorporated with NUFA for the primary immunization, to enhance the immune response. HSA-Cys-Gly-GnRH was dissolved in a sterile saline solution (0.9%, pH 7.2) and C. parvum was added (0.35 mg injection \(^{-1}\)). This aqueous phase was emulsified with NUFA oil in 50:50 proportions by forcing the liquids several times through a small orifice (double-ended 16-gauge steel needle between two syringes). The quality of emulsion formed was tested by its ability to form a droplet on water. The immunogen was prepared directly before immunization and administered at a final volume of 3 ml per animal.

All immunogens were administered s.c. at two sites in the neck-brisket area.

**Blood samples, body mass and testicular measurements**

Every 14 days throughout the experiment, blood samples were collected from all animals by jugular venepuncture and the animals were weighed. Individual average daily gains were calculated. Blood was centrifuged at 1600 \(g\) for 20 min and plasma was harvested and stored at \(-20^\circ C\) until assayed. Testicular measurements were recorded for each animal by the same person once every 28 days as follows: length and depth (widest section of testis from back to front) of each testis were determined using callipers and the circumference of the scrotal sac (containing both testes) was measured with a pliable measuring tape.

**Hormone assays and antibody titre determination**

Testosterone and LH concentrations and antibody titres were determined in all plasma samples. Testosterone concentrations were measured using a direct radioimmunoassay (Schanbacher and D’Occhio, 1982) that had been validated for use in our laboratory (Ronayne et al., 1993). The intra-assay coefficients of variation \((n = 10)\) were 4.1 and 10.8% for two plasma samples containing 5.19 and 34.6 nmol testosterone 1\(^{-1}\), respectively. The interassay coefficients of variation \((n = 10)\) for the same two samples were 11 and 15%, respectively. The sensitivity of the assay was 0.173 nmol testosterone 1\(^{-1}\).

Concentrations of LH were determined using a modification of the assay of Niswender et al. (1968). Briefly, the assay involved incubation of 200 \(\mu l\) of plasma for 24 h at 4°C in 300 \(\mu l\) PBS (pH 7.2) and 200 \(\mu l\) of antisera (NIADDK-antilH-I-l, AFP-192279) at a 1:600 000 dilution (raised in rabbits and donated by A. F. Parlow of NIDDK, CA). Next, 100 \(\mu l\) of 125I-labelled bovine LH [USDA(bLH-I-l, CAIFP-6000)] was added and all tubes were incubated for a further 20 h at 4°C. The bovine LH was iodinated using the iodogen method of Salacinski et al. (1981). The LH standard used was bovine-LH-B9 (NIH), which was supplied by NIDDK. Bound LH was separated from unbound LH by incubation with 100 \(\mu l\) of a second antibody [donkey anti-rabbit (A-SAC 1); Sac-Cel\(^{10}\), IDS Ltd, Bolton] for 30 min at room temperature. After centrifugation at 1600 \(g\) for 5 min, the supernatant was aspirated and the radioactivity of the pellet determined in a gamma counter. The intra-assay coefficients of variation \((n = 9)\) for this study were 6.3 and 11.9% for two samples containing 1.0 and 5.5 ng LH ml\(^{-1}\), respectively. The interassay coefficients of variation \((n = 6)\) for the same two samples were 10.5 and 14.5%, respectively. The sensitivity of the assay was 0.5 ng LH ml\(^{-1}\) plasma.

Antibody titres were determined by testing the binding of 125I-labelled GnRH (Amersham) in plasma using a radioimmuno-precipitation technique (Ciba Animal Health Research, St Aubin). This was carried out as follows: 100 \(\mu l\) 125I-labelled GnRH (10 000 c.p.m.) was added to 400 \(\mu l\) of five serial dilutions (1:40 to 1:640) of plasma. After an overnight incubation at 4°C, bound 125I-labelled GnRH was separated from unbound hormone by adding 500 \(\mu l\) of a 25% solution of polyethylene glycol (PEG; 8 kDa; Sigma Chemical Co) in PBS (pH 7.2) to each tube. The tubes were vortexed immediately, incubated at room temperature for 30 min and then centrifuged at 1600 \(g\) for 20 min. The supernatant was aspirated and the pellet resuspended in 500 \(\mu l\) of a 12.5% PEG solution. The tubes were incubated at room temperature for a further 20 min and then centrifuged at 1600 \(g\) for 10 min. The supernatant was aspirated and the radioactivity in the pellet measured in a gamma counter. The bound 125I-labelled GnRH proportion was determined for each plasma dilution and results are expressed as the percentage of total 125I-labelled GnRH bound at the 1:160 dilution of plasma. Nonspecific binding of 125I-labelled GnRH to factors in plasma other than GnRH antibodies was determined using normal control plasma. The mean nonspecific binding (for ten replicates) was only 0.4% at the 1:160 plasma dilution and was not subtracted from each individual titre measured during the experiment. The intra-assay \((n = 10)\) and inter-assay \((n = 20)\) coefficients of variation for a standard bovine antiserum plasma were 3.1 and 6.2% (at the 1:160 dilution), respectively.
Table 1. The numbers of GnRH-immunized bulls with antibody titres within a selected titre range (< 10% to > 70% binding at a 1:160 plasma dilution) 28 days after booster immunization and on day 140; the numbers of animals with a maximum antibody titre (reached at any time during the experiment) within each titre range are also given.

<table>
<thead>
<tr>
<th>Antibody titre range</th>
<th>28 days after booster (n = 48)</th>
<th>140 days after primary injection (n = 47)</th>
<th>When maximum titre was achieved</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10%</td>
<td>1 (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10–30%</td>
<td>7 (15)</td>
<td>6 (12)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>30–50%</td>
<td>20 (42)</td>
<td>21 (44)</td>
<td>12 (25)</td>
</tr>
<tr>
<td>50–70%</td>
<td>20 (42)</td>
<td>18 (37)</td>
<td>31 (65)</td>
</tr>
<tr>
<td>&gt; 70%</td>
<td>0 (0)</td>
<td>2 (4)</td>
<td>3 (6)</td>
</tr>
</tbody>
</table>

n: number of animals.

Statistical analyses

Data were analysed by ANOVA using both 3 (dose) × 2 (adjuvant) × 2 (booster interval) and 2 × 2 × 2 factorial (minus the 0.0 mg dose of conjugate) plans for a randomized complete block design. All data were analysed for each individual sampling date separately. Data were also analysed for two separate periods [period 1 (day 0–70) and period 2 (day 70–140)] and for the entire experimental period (day 0–140). The average daily gain (mm) in testes depth and length (mean of both the left and right testes measurements) and scrotal circumference were calculated. The effects of primary–booster interval on antibody titres and on testosterone and LH concentrations were calculated for 70 days immediately after the respective booster immunizations, whereas the effects of this interval on testes depth, length, scrotal circumference and average daily gain in body mass was calculated for the 84 days after the respective booster immunizations. Differences between specific treatments were determined using Fisher’s two-tailed test (Gill, 1978) and were considered statistically different when P < 0.05. Owing to infection with Mycobacterium tuberculosis, one animal that received 1.0 mg HSA-Cys-Gly-GnRH in NUFA with a primary–booster interval of 28 days was slaughtered on day 183. Missing values were estimated in the ANOVA.

Examination of testosterone concentrations in each animal was used to determine the most effective treatment combination. The period of consistently low testosterone concentrations in each animal was determined; that is, the greatest number of consecutive days during the experiment that testosterone concentrations were ≤1.73 nmol l⁻¹ (that is, the mean concentration of testosterone before the start of the puberal rise in testosterone in control bulls). A non-parametric statistical test (Mann–Whitney U test) was performed on the peak antibody titre data.

Correlation coefficients (r) were calculated for the comparison of the change in testes and body growth and the mean of all other variables over the experimental period. Correlation matrices using all 12 treatment combinations (n = 71), each separate group treated with a certain dose of HSA-Cys-Gly-GnRH (n = 23–24) and all animals treated with HSA-Cys-Gly-GnRH (n = 47) were prepared.

Results

Antibody titres

After immunization, all 48 GnRH-immunized bulls developed anti-GnRH antibodies; however, there was some variation in immune response among bulls (Table 1). Forty-six bulls had maximum antibody titres between 30 and 92% during the experimental period; however, for 31 of these animals the maximum titre was between 50 and 70%, whereas only two bulls had maximum titres of less than 30% binding. The overall mean maximum antibody titre achieved was 55.7%. Titres persisted in all bulls for the duration of the experiment and, at the end of the experiment (day 140), 41 bulls still had titres greater than 30% binding (Table 1).

There was a significant effect of dose of conjugate on antibody titres (Table 2 and Fig. 1). A lower (P < 0.05) overall mean antibody titre was obtained when 0.1 mg was used compared with 1.0 mg (29.6% versus 37.7% binding, respectively; pooled SED 2.55%). There was also an effect of primary–booster interval on antibody titres after the respective booster injections (Fig. 2a). All bulls reached their peak titre after their respective booster immunization. During the 70 days after respective booster immunizations, bulls with a primary–booster interval of 56 days had a higher (P < 0.05) mean titre than did those with an interval of 28 days (49% versus 42% binding, respectively; SED 3.2%). There was an apparent effect of adjuvant type on the rise of titre and eventual level of antibody titre (Fig. 2b). During period 1 (days 0–70) of the experiment, bulls given DEAE-D had higher (P < 0.05) mean titres than did those given NUFA (25% versus 18% binding, respectively; SED 2.7%). Specifically, titres were higher at days 28 and 42 and lower at day 140 in animals given DEAE-D than in those given NUFA (Fig. 2b).

LH and testosterone concentrations

The dose of conjugate used affected LH and testosterone concentrations during the experiment. Bulls immunized with 0.1 and 1.0 mg of HSA-Cys-Gly-GnRH had lower (P < 0.05) mean LH and testosterone concentrations compared with
Table 2. The effect of dose of the HSA-Cys-Gly-GnRH conjugate on mean plasma antibody titres, testosterone and LH concentrations, and the gain in live mass during the 140 day experiment in bulls

<table>
<thead>
<tr>
<th>Variable</th>
<th>0.0</th>
<th>0.1</th>
<th>1.0</th>
<th>Pooled SED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Antibody titres (% binding)</td>
<td>0.9a</td>
<td>29.6b</td>
<td>37.7c</td>
<td>2.55</td>
</tr>
<tr>
<td>Testosterone (nmol l⁻¹)</td>
<td>3.29a</td>
<td>2.18b</td>
<td>1.18c</td>
<td>0.336</td>
</tr>
<tr>
<td>LH (ng ml⁻¹)</td>
<td>1.90a</td>
<td>1.62b</td>
<td>1.56b</td>
<td>0.154</td>
</tr>
<tr>
<td>Mass gain (kg in 140 days)</td>
<td>114</td>
<td>107</td>
<td>113</td>
<td>4.1</td>
</tr>
</tbody>
</table>

abc Means within a row with different superscripts are significantly different (P < 0.05; two-sided test).

Fig. 1. The effect of dose of a GnRH analogue–human serum albumin (HSA-Cys-Gly-GnRH) conjugate (0.0, ●; 0.1, ▲; 1.0, ■ mg) on GnRH antibody titres (% binding at a 1:160 plasma dilution) in bull calves (n = 24 per dose group). *Within day, mean titre of animals immunized with the 1.0 mg dose is significantly different (P < 0.05) from that of animals immunized with 0.0 or 0.1 mg.

control bulls (Table 2). The mean durations of reduced LH and testosterone concentrations were longer in bulls immunized with 1.0 mg (56 and 98 days for LH and testosterone, respectively) compared with bulls immunized with 0.1 mg (14 and 56 days, respectively; Fig. 3). By day 140, mean testosterone concentrations had increased to values similar to those of control bulls in bulls immunized with 0.1 mg but not in bulls immunized with 1.0 mg (Fig. 3b). However, mean LH concentrations from day 112 to day 140 of the experiment

Fig. 2. Effects of (a) primary–booster interval of (●) 28 days or (■) 56 days and (b) adjuvant type, (●) non-ulcerative Freund’s adjuvant, or (■) diethylaminoethyl-dextran, on GnRH antibody titres (% binding at a 1:160 plasma dilution) in bull calves (n = 24 per group) immunized against 0.1 or 1.0 mg of a GnRH analogue–human serum albumin (HSA-Cys-Gly-GnRH) conjugate. Arrows indicate times of booster injection. *Within day, means are significantly different (P < 0.05).
were not significantly different \((P > 0.05)\) in bulls immunized with 0.0, 0.1 or 1.0 mg of conjugate (Fig. 3a).

There was an effect of primary–booster interval on LH and testosterone concentrations on specific dates after respective booster (Fig. 4). During the 70 days after each respective booster, bulls boosted 56 days after the primary injection had lower \((P < 0.05)\) LH concentrations \((1.1 \text{ versus } 1.4 \text{ ng ml}^{-1})\), respectively; \(\text{SE} \pm 0.07\) than did bulls boosted 28 days after the primary injection, but testosterone concentrations were similar in both groups of bulls (Table 5).

There was an overall effect of type of adjuvant on testosterone but not on LH concentrations. Bulls receiving DEAE-D had lower \((P < 0.05)\) mean testosterone concentrations than did bulls receiving NUFA (Fig. 5).

Examination of testosterone concentrations showed that 18 of 48 immunized bulls had testosterone concentrations \(\leq 1.73 \text{ nmol l}^{-1}\) for a mean of 126 days. The other 30 bulls had periods of suppressed testosterone ranging from 28 to 112 consecutive days. The bulls immunized with 1.0 mg GnRH conjugate had the greatest \((P < 0.05)\) consecutive number of days with low testosterone concentrations and achieved the highest \((P < 0.05)\) mean maximum titre during the experiment than either the bulls treated with 0.1 mg GnRH conjugate or control bulls (Table 3). Consequently, throughout the duration of the experiment, mean testosterone concentrations in the immunized bulls \((n = 23)\) were negatively correlated \((P < 0.001)\) with mean antibody titres \((r = -0.48)\).

**Testicular growth and animal performance**

Immunization of bulls against the GnRH conjugate reduced \((P < 0.05)\) testes growth during the experiment. Specifically,
Fig. 5. The effect of adjuvant type (non-ulcerative Freund's adjuvant, •: diethylaminoethyl-dextran, ■ on mean testosterone concentrations of bull calves (n = 24 per adjuvant group) actively immunized against 0.1 or 1.0 mg of a GnRH analogue–human serum albumin (HSA-Cys-Gly-GnRH) conjugate. *Within day, mean testosterone concentrations are significantly different (P < 0.05).

Table 3. Mean maximum antibody titres achieved during days 0–140, and mean number of consecutive days when testosterone concentrations were ≤1.73 nmol l⁻¹ for each of the main effects of HSA-Cys-Gly-GnRH immunization of in bulls: conjugate dose, adjuvant and primary–booster interval.

<table>
<thead>
<tr>
<th>Main effect</th>
<th>Maximum antibody titre</th>
<th>Number of days when testosterone ≤1.73 nmol l⁻¹</th>
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<tr>
<td>Dose of HSA-Cys-Gly-GnRH (mg)</td>
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<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.82a</td>
<td>32.7a</td>
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<tr>
<td>0.1</td>
<td>51.3b</td>
<td>66.5b</td>
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<td>1.0</td>
<td>60.2c</td>
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<tr>
<td>SED</td>
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<td>NUFA</td>
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<td>DEAE-D</td>
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<td>Primary–booster interval (days)</td>
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<td>TLG (mm day⁻¹)</td>
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<tr>
<td>TDG (mm day⁻¹)</td>
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<td>0.13b</td>
<td>0.09c</td>
<td>0.010</td>
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<tr>
<td>SCG (mm day⁻¹)</td>
<td>0.73a</td>
<td>0.57b</td>
<td>0.43c</td>
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<td>ADG (kg day⁻¹)</td>
<td>0.81</td>
<td>0.77</td>
<td>0.81</td>
<td>0.034</td>
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</table>

*Means within a row with different superscripts are significantly different (P < 0.05; two-sided test).

The testes of the bulls boosted 56 days after the primary injection grew more slowly (P < 0.05) than did the testes of bulls boosted 28 days after the primary injection (Table 5). The type of adjuvant used also affected testes growth; specifically, the bulls immunized with DEAE-D had reduced (P < 0.05) testicular growth compared with bulls immunized with NUFA (Table 5).

The overall experimental mean testes depth and length and scrotal circumference for the GnRH-immunized bulls (n = 47) were negatively correlated (P < 0.05) with antibody titre (r = -0.43, -0.49 and -0.39, respectively) and positively correlated (P < 0.05) with mean testosterone concentrations (r = 0.71, 0.76 and 0.76, respectively) and body mass (r = 0.48, 0.37 and 0.45, respectively).

There was no effect of dose of conjugate on average daily gain of testes mass. Bulls immunized with 0.1 and 1.0 mg GnRH conjugate had similar liveweight gain and average daily gain compared with the control bulls (Tables 2 and 4). There was also no effect of adjuvant type or primary–booster interval on average daily gain (Table 5).

**Discussion**

This study provides information on how dose of a GnRH analogue conjugated to HSA, adjuvant type and primary–booster interval can affect immune, endocrine, testicular and growth responses in bulls following active immunization against GnRH. The HSA-Cys-Gly-GnRH conjugate used was an effective immunogen when administered in either 0.1 or 1.0 mg quantities, with either NUFA or DEAE-D adjuvant and with boosters at either 28 or 56 days after primary immunization. All bulls receiving the GnRH immunogen developed anti-GnRH antibodies, and titres persisted for the duration of the experiment (140 days). Bulls immunized against GnRH had extended periods of reduced plasma LH and testosterone concentrations and testes growth, but there was no adverse effect on body growth. DEAE-D was the most effective adjuvant, inducing higher titres earlier in the experiment than did NUFA and consequently causing greater suppression of testes growth and testosterone concentrations. Both booster intervals used caused an immediate rise in titres and subsequent suppression of LH and testosterone concentrations and testes growth.

Table 4. The effect of dose of the HSA-Cys-Gly-GnRH conjugate on average daily gain of testes length (TLG) and depth (TDG) and of scrotal circumference (SCG) and body mass (ADG) of immunized bulls (n = 24 per group) over the experimental period.
The immune response achieved in this study was relatively consistent and prolonged in contrast to the short-lived and variable immune response achieved in bull calves immunized against GnRH conjugated to human (Robertson et al., 1979, 1981, 1982, 1984) or bovine serum albumin (Jeffcoate et al., 1982), ovalbumin or horse albumin (Gonzalez et al., 1990). In these studies, the increased antibody titres were low or occurred in only 50% of the immunized animals; in addition, in responding animals titres were often short lived or only sustained by repeated immunizations.

The highest anti-GnRH antibody titres were achieved in the bulls immunized with 1.0 mg conjugate. This finding is in agreement with data presented by Carson et al. (1992), who reported that 1.0 mg GnRH conjugate given to prepubertal bulls elicited a better antibody response than did either 0.05, 0.25 or 4.0 mg of conjugate. Similar anti-GnRH antibody titres to those in our study were achieved in bulls and steers immunized against 2 or 5 mg equivalents of a synthetic GnRH covalently conjugated to keyhole limpet haemocyanin (1 mg equivalent contained 0.65 mg GnRH) with both single and primary plus booster immunization regimens (Adams and Adams, 1992; Adams et al., 1993); however, the immunogen included Freund’s complete adjuvant in the primary immunizations. The most effective immunosuppression of bulls in this study was achieved using 1.0 mg conjugate, as judged by the number of days that testosterone was maintained at values below 1.73 nmol l⁻¹. Similar immunosuppression (testosterone < 1.04 nmol l⁻¹ for 119 days) was achieved by Lobley et al. (1992) after a booster injection in 50% of bulls immunized with an octapeptide conjugated to egg albumin.

The mean maximum titre achieved in this study was comparable to maximum titres achieved by Jeffcoate et al. (1982) and Adams et al. (1993) in bulls immunized before puberty; that is, approximately 50% binding at a serum dilution of 1:100 for both studies. This study shows a prolonged period of suppressed testosterone secretion and testes growth in all GnRH-immunized bulls. A similar suppression of testosterone secretion at between 4 and 10 months of age was seen in a study by Robertson et al. (1982) in two groups of animals. However, even in the presence of suppressed testosterone secretion, animals with titres of less than 50% binding at 1:1000 dilution were termed ‘non-responders’. In the extreme, it has been reported that animals with titres as low as 10% binding at a 1:10 serum dilution have reduced testosterone concentrations (Gonzalez et al., 1990) compared with those of normal bulls. However, in the study by Robertson et al. (1982), no comparison with normal bulls was made. Our data indicate that peak titres of less than 50% and greater than 18% binding at a 1:160 plasma dilution can suppress testosterone concentrations for up to 126 days and testes growth for up to 140 days after primary injection. However, it was not possible to select an absolute minimum titre or threshold titre necessary to achieve a definite period when responses similar to those after castration could be recorded.

Because of its effective adjuvant activity, most of the more successful active GnRH immunization studies have included Freund’s complete adjuvant in the vaccine formulation. However, this adjuvant is not suitable for a practical GnRH immunization regimen because it is oil based. DEAE-D is soluble in water which eliminates the need for a non-degradable mineral oil base. It has been shown to elicit substantial immune responses in pigs against the foot and mouth disease virus (Wittmann et al., 1970, 1972) and in heifers against GnRH (D’Occio et al., 1992). In addition, a combination of DEAE-D and a mineral oil elicits a good immune response against GnRH in heifers, ewes and rams (Hoskinson et al., 1990). The higher titres achieved in the DEAE-D-treated animals in this study demonstrate that DEAE-D may be a suitable alternative to oil-based adjuvants.

The normal pubertal rise in testosterone concentrations and testes size occurred in control bull calves in our experiment (MacMillan and Hafs, 1968, 1969; Rawlings et al., 1972; Karg et al., 1976) between 4 and 7 months of age, whereas all GnRH-immunized bull calves had periods of suppressed testes growth, and plasma LH and testosterone concentrations. Testosterone concentrations in the control animals at 6–7 months of age were comparable to those in previous studies with peri-pubertal bulls (McCarthy et al., 1979; Adams and

![Fig. 6](image-url)
Adams, 1992). Reduced testes size and LH and testosterone concentrations due to anti-GnRH antibody titres have been reported from studies with bulls (Robertson et al., 1979, 1981, 1982, 1984; Jeffcoate et al., 1982; Carson et al., 1992) and rams (Jeffcoate et al., 1982; Schanbacher, 1982; Chaffaux et al., 1985; Chase et al., 1988; Goubau et al., 1989a). However, in most previous studies, sufficient titres to elicit or maintain these biological effects in all animals were generally not achieved without at least three immunizations (Jeffcoate et al., 1982; Gonzalez et al., 1990). The reduction in LH noted in our study was temporary for both the 0.1 and 1.0 mg doses of GnRH conjugate; however, testosterone concentrations in bulls immunized with 1.0 mg remained suppressed until at least 140 days after the primary injection, as did testicular growth in all GnRH-immunized bulls. These facts indicate the biological significance of the titres achieved in this study.

Robertson et al. (1982) demonstrated periods of reduced testosterone secretion and testes growth of approximately 12 and 15 weeks, respectively, in five ‘responders’ out of ten GnRH-immunized bulls. They suggested (based on handling) that these animals were easier to manage than normal bulls. Unlike the results reported here, Robertson et al. (1979, 1982) noted that the pubertal rise in both testosterone concentration and testes size had begun before immunization took effect. By immunizing prepubertal bulls four times, Jeffcoate et al. (1982) prevented the pubertal increase in testes size for at least 20 weeks after primary immunization. More recently, Lobley et al. (1992) and Adams et al. (1993) also observed a reduction in testosterone concentration and testes size in bulls immunized prepubertally. However, these immunogens contained NUFA and Freund’s complete adjuvant, respectively.

There was a negative correlation between antibody titres and testosterone concentrations and testes size; however, in the latter stages of the experiment, both LH and testosterone concentrations increased and testes size began to increase even in the presence of high antibody titres. Adams and Adams (1992) reported a lack of correlation between antibody titre and biological immunity in studies with GnRH. Wittman et al. (1972) reported a similar lack of correlation with anti-viral vaccines. A compensatory increased release of GnRH or an increased sensitivity of the GnRH receptor may explain the resumption of normal LH and testosterone secretion in the presence of anti-GnRH antibodies. However, there is some evidence to suggest that antibody titres are not biologically active after they reach a plateau (M. Finnerty et al., unpublished). Further studies are required to explain this phenomenon.

Gonadal steroids play a critical role in animal growth and development (Schanbacher, 1984b). However, even with reduced testes size and testosterone concentrations, there was no effect of conjugate dose, adjuvant or primary–booster interval on growth rate. The lack of effect of GnRH immunization on growth rate in bulls observed in our study is in agreement with studies with bulls by Gonzalez et al. (1990), Adams and Adams (1992), Lobley et al. (1992) and Adams et al. (1993).

In conclusion, a relatively consistent and persistent biological effect on the hypothalamic–pituitary–testicular axis of all GnRH-immunized bulls was achieved without an adverse effect on growth rate. In addition, active immunization of bulls with 1.0 mg of a HSA-Cys-Gly-GnRH conjugate in combination with DEAE-D adjuvant and a primary–booster interval of 56 days was a successful treatment combination and may be the optimum immunization protocol for future GnRH immunization studies in bull calves.
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