

Active immunization against adrenocorticotrophic hormone (ACTH) in grazing lambs suppresses the immune response to a GnRH antigen but does not alter growth

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(Revised MS received 10 March 2004)

SUMMARY

This experiment examined the effects of immunizing against ACTH (adrenocorticotrophic hormone) and GnRH (hypothalamic peptide gonadotrophic releasing hormone) on growth and carcass composition of entire ram lambs from 6 to 35 weeks of age. There were four experimental groups (each of 17 animals) in a 2 × 2 factorial design: (1) a non-immunized control group, (2) immunized against ACTH, (3) immunized against GnRH and (4) immunized against both antigens. After booster injections, experimental lambs exhibited antibody titres to the two antigens presented, resulting in significantly lower cortisol and testosterone levels ($P < 0.001$) in peripheral blood. The fall in blood cortisol levels in response to ACTH immunization was later matched by a similar decline in control animals to a point where they were not different.

In GnRH-immunized animals, antibodies disrupted the pituitary-gonadal axis, thereby impacting severely on testes development in growing lambs to the degree that by day 119 their testes were smaller than at the commencement of the experiment. Towards the end of the growth phase of the animals, GnRH-immunized lambs had significantly lower body weights at day 189 ($P < 0.05$) and day 204 ($P < 0.01$) while ACTH-immune lambs were not different in weight from control animals. Also, GnRH-immunized lambs were found to have greater GR measurement ($P < 0.05$). Although ACTH immunization was capable of lowering cortisol for a brief period during the current experiment, the results were variable and detrimental to the efficacy of the immuno-castration vaccine (GnRH).

INTRODUCTION

In farm animals, the production of glucocorticoids by the adrenal cortex represents an important adaptive response mechanism that may also have implications for animal productivity. Glucocorticoids have been shown to be responsive to stress, with energy mobilization and anti-inflammatory effects (Bjorntorp *et al.* 1990). In addition, there is evidence that glucocorticoids have effects on growth (Wehrenberg *et al.* 1990). The pervasive influence of these adrenal steroids on

the stress response commences with their ability to initially prime the defence mechanisms of the animal, followed by their ability to prevent stress-activated defence mechanisms from 'overshooting' thus causing tissue damage, and then by their ability to enhance the initial acute physiological adjustments induced by effector molecules of the endocrine-immune-sympathetic neural axis (Munck *et al.* 1984; Sapolsky *et al.* 2000). The chronic elevation of circulating glucocorticoids in both ruminant and non-ruminant species has been associated with a decrease in growth rate (Purchas *et al.* 1980) which is accompanied by muscle wastage (Tomas *et al.* 1979) and the accumulation of body fat. This latter

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response appears to be the net result of the opposing influences of insulin and the glucocorticoids in regulating the partitioning of energy substrate (Strack *et al.* 1995).

The immuno-suppressive and anti-inflammatory effects of high levels of glucocorticoids in mammals and other species have been well documented and are known to be mediated through a decrease in pro-inflammatory cytokine levels (Baxter & Rousseau 1979; Liddle 1981; Sapolsky *et al.* 2000).

Since glucocorticoid hypersecretion appears to suppress growth performance and immune function, the long-term suppression of secretory peaks of these hormones provides a potential target to boost growth efficiency and animal health. Our previous studies (Wynn *et al.* 1994) have shown that the induction of autoimmunity to adrenocorticotrophic hormone (ACTH) using an ACTH (1-24):human serum albumin (HSA) conjugate in Freund's adjuvant in grazing sheep can decrease carcass fatness and increase muscle yield.

In this study we investigated the impact of ACTH immunization on the humoral immune response in grazing lambs post-weaning, since it was well recognized that cortisol hypersecretion was negatively correlated with antibody titres directed at foreign antigens (Cummins & Brunner 1991). We used a second production-related antigen to assess humoral immune responsiveness, the anti-GnRH vaccine used to immunocastrate animals (Hoskinson *et al.* 1990).

From previous studies, it was hypothesized that lowering circulating blood cortisol by ACTH immunization may lead to improved antibody titres to a subsequent immunization against GnRH. A further objective was to determine the impact of the immunization treatments on growth rates and carcass composition of young lambs under extensive grazing.

MATERIALS AND METHODS

Animals and treatments

Ram lambs (Dorset × Border Leicester-Merino) were grazed with their dams on phalaris/white clover pasture during 1993–94 at the F. D. McMaster Laboratory at Armidale, NSW, Australia (latitude 30° south, longitude 151° east, altitude 1046 m) and weaned at 100 days of age.

The lambs ($n=68$) were allocated to one of four groups ($n=17$ per group) based on stratified live weights in a 2 × 2 factorial design.

Treatments were as follows:

- (1) ACTH immunization: At the commencement of the experiment (day 1), lambs received a primary immunization with ACTH (1-24):ovalbumin (OA) conjugate (0.5 mg) emulsified in Freund's complete adjuvant (1 ml: Sigma Chemical Company, St Louis) and 1 ml of sterile saline, injected

intramuscularly at one site in each hind leg at 6 weeks of age. Three booster immunizations were administered on days 31, 57 and 88, using the same antigen emulsified in Freund's incomplete adjuvant (1 ml) and saline (1 ml) as above.

- (2) GnRH immunization: Lambs were immunized with a GnRH:HSA conjugate (1 mg). The conjugate was emulsified using a mixture of a solution containing 5% Diethylaminoethyl (DEAE)-dextran (Sigma) in a 50% water-in-mineral oil emulsion and injected into two sites as per group 1. The primary injection was given on day 35 and a single booster injection given on day 84.
- (3) Lambs received both the ACTH and GnRH immunization treatments as described above.
- (4) Control lambs received the maximum number of injections of the vehicle (HSA or OA) plus the respective adjuvants on days 1, 31, 35, 57, 84 and 88.

Animals were weighed monthly throughout the trial and at the same time an assessment of testicular volume and weight was determined on entire animals to quantify unbound gonadotrophin levels in response to GnRH immunization. A set of 10 egg-shaped graduated wooden beads ranging in diameter from 18 to 58 mm and calibrated to testis weight was used to assess testis development in the live animal (Oldham *et al.* 1978). A strong correlation between testicular diameter and sexual maturity and activity has been demonstrated previously in sheep and cattle (Brown *et al.* 1994).

Blood samples

Samples were collected from all animals by jugular venepuncture immediately before and 7 days after each vaccination. Samples were placed on ice immediately and centrifuged (1200 g for 15 min) prior to the separation of plasma, which was stored at –20 °C pending analysis.

Immunogens

ACTH (1-24) (Ciba Geigy, Basel, Switzerland) in the free acid form was conjugated to OA (Sigma) using 1-ethyl-3-dimethylamino propyl carbodiimide in a weight ratio of 1:1:10 (Goodfriend *et al.* 1964). The reactants were stirred at 4 °C for 16 h and the products filtered and dialysed against 0.9% saline for a further 48 h. The products were recovered by lyophilization and stored until reconstituted for injection.

GnRH (1-10) in the free acid form was conjugated to human serum albumin (HSA) using the same conjugation chemistry as for ACTH.

Plasma antibody titres

Blood samples collected throughout the experiment from the immunized lambs were analysed for ACTH

and GnRH antibody titres using ELISA. Microtitre plates (96 well round bottom, Dynatech: Dynatech Laboratories, Virginia) were coated with an ACTH:gelatin (1:2, w:w) conjugate or GnRH:gelatin (1:2, w:w) in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) at a concentration of 0.03 mg/ml (100 µl) and sealed for 18 h at 4 °C. Plates were washed three times with PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). Wells were blocked with 0.3% gelatin (Type A: Sigma) in PBS containing 0.05% Tween 20 (BDH Chemicals, Sydney). Plasma samples were serially diluted (100 µl per well) with an internal standard being included on each plate and incubated for 1 h at 23 °C. Plates were washed with PBS-Tween 20 (3 times) and the enzyme substrate p-nitrophenyl-phosphate added (100 µl of a 1 mg/ml solution in 0.5 mM MgCl₂·6H₂O, 9.7% diethanolamine pH 9.8; Sigma). The reaction was allowed to develop for 30 min in the dark and was stopped with 3 M NaOH (50 µl/well) and the optical density of wells recorded with a plate reader (Dynatec Laboratories, Virginia). The between-assay coefficient of variation was 21%.

Plasma cortisol

Cortisol was assessed by radioimmunoassay using a modification of the method of Foster & Dunn (1974). In brief, borate buffer (760 µl, 0.02 M, pH 7.6) with gelatin (0.001%: 300 bloom, Sigma) and sodium azide (0.5%, Sigma) was heated with plasma (20 µl) or cortisol standard (20 µl, Sigma) to 60 °C for 1 h in 12 × 75 mm glass tubes. Cortisol antisera (100 µl) and tracer (6000 cpm/50 µl) were added on cooling, prior to incubation for 18–24 h at 4 °C. A charcoal suspension (100 ml: 0.025% activated charcoal, Ajax, Sydney) containing dextran T70 (0.0025%) (Pharmacia) was subsequently added and incubated for 1 h at 4 °C. Tracer not bound to antibody was separated after centrifugation at 1000 g at 4 °C for 15 min by aspiration of the supernatant. The sensitivity of the assay was 0.8 ng/ml while the between- and within-assay coefficients of variation were 6.1 and 2.9% respectively.

Testosterone

Plasma testosterone concentrations were measured in duplicate using a single antibody technique (Garnier *et al.* 1978). The samples and standards (20 µl) were extracted for 5 min with 2 ml of toluene:hexane (2:1, v:v) and the solvent phase was evaporated to dryness under N₂. An antiserum raised in sheep against testosterone-3-carboxymethyloxime:BSA (M. Wong and R. I. Cox, CSIRO, Division of Animal Production, Blacktown, NSW) was used at a dilution of 1:60 000. Crossreactivity with dihydrotestosterone, 4-androstene-3β, 17β-diol and androstenedione was

31, 30 and 1.3% respectively. All oestrogen and progestagen compounds had <1% crossreactivity and the sensitivity of the assay was 0.1 ng/ml.

Statistical analyses

Experimental data were analysed using a Generalized Least Squares Program (REG, Version 90.12; NSW Agriculture & Fisheries) using standard procedures for repeated measures analysis of variance, one-way analysis of variance, Student's *t*-test to compare means and regression analysis. The live weight of lambs and testes measurements were analysed in a model for repeated measures, which included terms for ACTH and GnRH immunization, initial live weight and all first order interactions. After dropping non-significant ($P > 0.05$) interactions from the model, the simplest significant ($P < 0.05$) model was incorporated in the statistical analysis. Carcase data were analysed fitting hot carcase weight into the model as a covariate, in order to adjust for differences in final live weights at slaughter.

RESULTS

ACTH antibody titres

Large increases in antibody titres were observed in response to the booster immunization on day 39, with a 6-fold greater response being observed in the group of animals immunized against ACTH just prior to receiving the GnRH antigen (Fig. 1). These declined, however, to levels similar to that of the ACTH immune group by day 130, which were consistently lower than those observed in the double-immunized animals for the duration of the experiment.

Plasma cortisol

Mean blood cortisol concentrations were between 65 and 80 ng/ml at the start of the experiment in all animals and declined thereafter at each subsequent sampling in all groups (Fig. 2). However, both ACTH and ACTH+GnRH immunized lambs had lower blood cortisol concentrations than control lambs following the booster immunization against ACTH at day 31, an effect that persisted to the end of the study.

GnRH antibody titres

Elevated anti-GnRH antibody titres were detected 7 days after the booster immunization (Fig. 3). Antibody titres rose through to day 93 and decreased thereafter to the completion of the study on day 200. These did not vary significantly between the ACTH and ACTH+GnRH immunized animals.

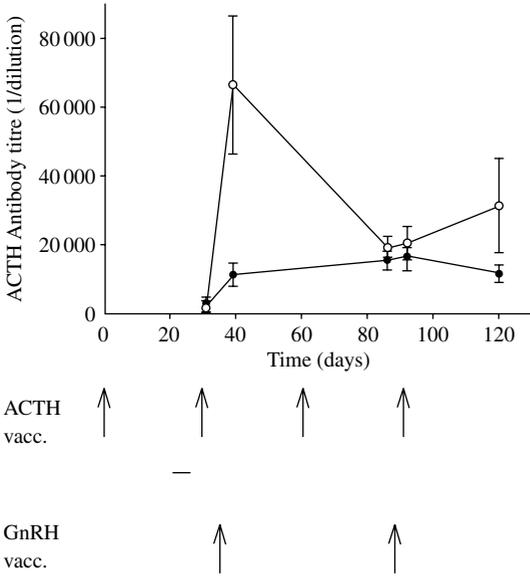


Fig. 1. ACTH antibody titre for lambs actively immunized against ACTH (●) or against ACTH and GnRH (○). Arrows denote time of vaccination.

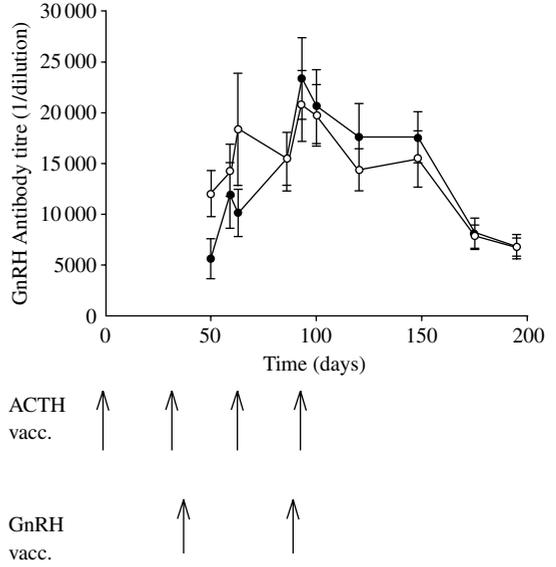


Fig. 3. GnRH antibody titre levels for lambs immunized against GnRH (●) or a combination of immunogens (○). Arrows denote time of vaccination.

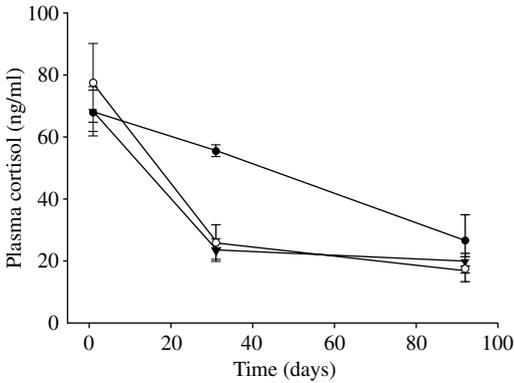


Fig. 2. Plasma cortisol levels in male lambs either immunized against ACTH (○), ACTH+GnRH (▼) or non-immunized controls (●).

Plasma testosterone

GnRH immunization ablated circulating testosterone concentrations at day 200 to a level below the sensitivity of the assay (0.1 ng/ml). ACTH immunization alone had no effect on testosterone concentrations, however, the combined ACTH+GnRH immunization resulted in a significant but partial reduction of testosterone secretion (Fig. 4).

Testis development

The combined estimated weight of testes for the intact control rams was 20.6 ± 3.45 g at the commencement

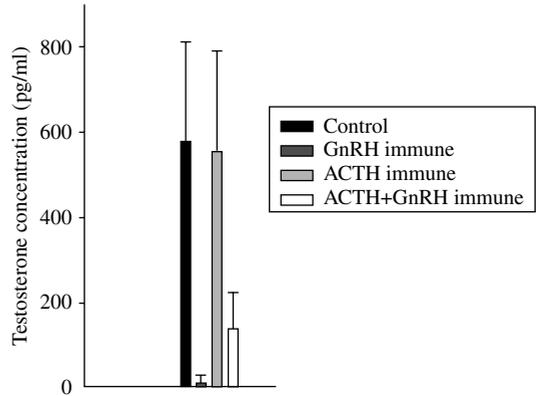


Fig. 4. Plasma testosterone levels for ram lambs at the completion of the experiment. Testosterone levels for GnRH immune lambs were below the sensitivity of the assay. GnRH and ACTH+GnRH immunization significantly reduced circulating testosterone levels in lambs ($P < 0.001$).

of the trial and increased to a maximum size of 255.1 ± 16.09 g at day 175 (Fig. 5). ACTH immunization did not affect the normal testicular development in these young ram lambs.

In contrast, from day 50 there was a highly significant difference ($P < 0.001$) between the size of testes from animals immunized against GnRH and the lambs from other treatment groups. When adjusted to initial testis size, the testes from the GnRH

Table 1. Least square means for live weight (kg) for experimental ram lambs over the duration of the experiment. At any day values with differing superscripts are significantly different ($P < 0.05$) or xy ($P < 0.01$)

	Control	ACTH immune	GnRH immune	ACTH + GnRH immune	Average standard error
Day 1	11.1	11.0	11.0	11.1	0.15
Day 14	14.0	13.4	13.2	13.7	0.19
Day 44	20.3	20.0	19.7	19.9	0.37
Day 69	25.9	25.4	25.0	25.0	0.58
Day 98	31.2	30.6	30.3	29.9	0.6
Day 133	32.1	31	30.3	30.3	0.77
Day 162	36.3	36.1	34.4	34.8	0.68
Day 189	39.2 ^a	39.3 ^a	37.0 ^b	37.1 ^a	0.72
Day 204	40.0 ^c	41.3 ^c	38.3 ^{xy}	38.6 ^d	0.75

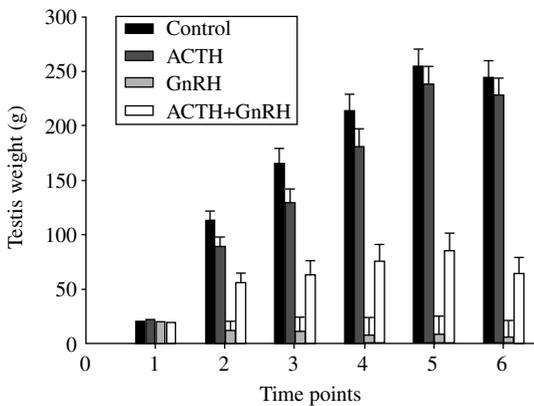


Fig. 5. Estimated weights of combined testes adjusted to initial testis weight for entire male lambs. GnRH immunization significantly reduced testis development in growing lambs ($P < 0.001$). Time points correspond to D38, D100, D119, D148, D175 and D190 respectively.

immunized rams regressed over time. However, in the ACTH + GnRH immune rams this regression was not of the same magnitude.

The final testis measurement recorded at the completion of the growth period (day 190) was plotted against the weight of testes measured at slaughter, resulting in a significant linear regression relationship.

$$Y = -4.746 + 0.781X$$

$$(\text{coefficient of determination} = 0.966)$$

Although there was a tendency to under-estimate the actual size of the smaller testes, the above regression demonstrated a strong relationship between estimated and actual testis weight. There was a negative correlation between estimated testes weight and GnRH antibody titre from day 100 to day 190. The relationship, however, was poor and non-linear, mainly due to lack of normality of the testes data.

Growth

The live weight of lambs on day 1 was 11.1 ± 0.75 kg. Animals attained commercial live weights at the time of slaughter (day 204), although the growth path was disrupted between days 100 and 150, during which time live weight did not change due to poor seasonal conditions resulting in lower pasture quantity and quality (Table 1). Throughout the 7-month study, there was no significant difference in body weight, or daily gain between the ACTH immunized animals and the non-immunized control animals.

In contrast, GnRH immunization significantly reduced live weight ($P < 0.05$) at day 189 and this decrease assumed greater significance ($P < 0.01$) at the completion of the experiment at day 204 (Table 1). Although there was a tendency for GnRH immune ram lambs to grow more slowly prior to day 162, the differences were not significant ($P > 0.05$). When adjusted using initial live weight as a covariate, there was a 2.2 kg difference between the non-vaccinated control group and the GnRH immunized group at the completion of the experiment.

Carcase composition

There was a tendency for GnRH immunization to depress both fasted slaughter weight and carcase weight (Table 2), but this effect did not attain statistical significance. Neither ACTH or GnRH vaccine affected kidney fat, but there was a significant interaction ($P < 0.05$) between the two vaccination treatments on omental fat content, suggesting that these depots varied in weight between the treatments depending on carcase weight. A significant effect of GnRH immunization ($P < 0.05$) was observed on the level of omental fat and on the depth of total tissue measured over the 12th rib at a point 110 mm ventrally from the vertebra (GR) (Table 2). Other carcase parameters (fat depth over 12/13 rib) in this same treatment group tended to exhibit more fat, although this failed to achieve significance.

Table 2. *Least square means for pre-slaughter live weight and carcass traits for ram lambs immunized against ACTH, GnRH or a combination of immunogens, after adjustment for the appropriate covariate. Data were analysed using a log transformed model and predicted means calculated by taking the antilog. Values with differing superscripts are significantly different ($P < 0.05$)*

	Control	ACTH immune	GnRH immune	ACTH + GnRH immune	Average standard error
Live weight at slaughter (kg)	39.8	40.7	37.6	38.5	1.46
Hot carcass weight (kg)	15.8	15.7	15.3	15.3	0.59
Kidney fat (g)*	272	242	302	269	26
Omental fat (g)*	454 ^d	424 ^d	545 ^c	508	31
Fat depth 12/13 Rib (mm)*	1.85	1.74	2.02	1.90	0.19
GR (mm) *	5.19 ^b	5.22	6.24 ^a	6.27 ^a	0.42
Eye muscle area (cm ²)*	9.40	9.30	9.69	9.59	0.37

* Treatment means adjusted to the same hot carcass weight.

DISCUSSION

In the current experiment, ACTH immunization proved to be detrimental to the immunological response to the GnRH immunization. Whilst growth rates were not affected by ACTH immunization, they were reduced by immunization against GnRH. Successful immunomodulation of the hypothalamic-pituitary-adrenal axis impacted on the biological effectiveness of the humoral response to the GnRH antigen in these experimental ram lambs. Despite the fact that antibody titres were similar in both groups, clearly the active GnRH immunization, when administered simultaneously with an ACTH antigen, was not as effective as GnRH immunogen administered alone. Earlier research has demonstrated a strong negative relationship between GnRH antibody titres, testes weight and testosterone status (Hoskinson *et al.* 1990). An interaction between ACTH and GnRH immunization was evident from the intermediate testis size and circulating testosterone status observed in the double-immune animals relative to the GnRH immune and control animals.

Grazing ruminants are subjected to a range of stressors that compromise protein accretion, immune function and therefore productivity, effects that are most likely associated with the chronic elevation in circulating glucocorticoids. The treatment of lambs with ACTH decreased the circulating T lymphocyte population (Kuhlman *et al.* 1991) while humoral responses to ovalbumin were suppressed by the administration of the synthetic glucocorticoid, dexamethasone, also in lambs (Minton *et al.* 1991).

Possible reasons for the differing results in the current study could be due in part to previous studies failing to characterize the effectiveness of antibodies to immunoneutralize the target antigen. The antigenic sites on the hypervariable loops on the immunoglobulin molecules are capable of generating a

network of idiotypic antibodies, some of which may mimic the original antigen. These anti-idiotypes may actually bind to and exert agonist activity on the GnRH receptor. Such effects have been identified using affinity purified anti-ACTH antibodies from sheep actively immunized against ACTH in adrenocortical cells *in vitro* (Shahneh 1995). Similar anti-idiotypes are associated with a number of endocrine pathologies including type 1 diabetes (Casiglia *et al.* 1991).

Alternatively an elevation in other peptide hormones derived from increased pro-opiomelanocortin (POMC) expression due to ACTH immunization (Wynn *et al.* 1994) may have maintained in part testicular steroidogenic activity. Such a response is most likely due to a reduction in the central negative feedback of either ACTH or glucocorticoids. POMC from both pituitary and testicular origin could have been involved here since this protein is processed to generate the same family of peptides in each of these tissues (Bardin *et al.* 1984).

β -endorphin is not likely to play such a direct role in view of its inhibitory effect on testicular steroidogenesis (Akibami & Mann 1996), although an earlier report suggested a facilitatory role for this peptide (Bardin *et al.* 1984). Although the μ -opioid receptor, the class to which β -endorphin transduces its bio-activity, and the δ -receptor were initially identified on T cells using functional assays (Wybran *et al.* 1979), full length cDNAs have also been identified for the α and δ opioid receptors in the circulating lymphocyte population (Belkowski *et al.* 1995; Sedqi *et al.* 1996).

It is possible that these receptors mediated the suppression in antibody production induced by either α -endorphin or Met and Leu-enkephalin as reported by (Johnson *et al.* 1982). Since the enkephalins are derived from pre-pro-enkephalin and not POMC, they are unlikely to be causal elements here. In contrast

α -endorphin results from the cleavage of the carboxyl terminal 5 amino acids of β -endorphin of POMC origin and is found in many peripheral tissues, although not the pituitary (Lebouille *et al.* 1986). The role of Sertoli cell-derived α MSH is similarly obscure, although it appears to stimulate aromatization of testosterone to oestrogen (Boitani *et al.* 1989) most likely without affecting Leydig cell steroidogenesis. The hormone initiating the hypothalamic-pituitary-adrenal cascade, corticotrophin releasing hormone (CRH), which is most likely upregulated in response to ACTH immunization, is also expressed in the testicular Leydig cells, yet any elevation in its expression most likely would have suppressed steroidogenesis (Dufau *et al.* 1993).

We hypothesized that the modulation of the secretion of glucocorticoids might improve the humoral immune response to the GnRH antigen. It is conceivable that the decreased effectiveness of the GnRH antibody population in the ACTH-immune animals may be due in some way to an adrenal insufficiency. Although enhanced inflammatory responses are characteristics of this physiological state, for example in both the Lewis rat (Sternberg *et al.* 1989; Frey *et al.* 1991) and in Addison's disease, its impact on chronic humoral immunity is not well defined. Yet the circulating cortisol concentrations in the ACTH-immune animals were not lower than the basal concentrations found in control animals, suggesting that this may not be a causal factor.

The significantly lower live weight in the GnRH immune male lambs as compared with the control males and ACTH immune lambs was most likely attributable to depressed plasma androgen levels post-immunization. Of note, was the significantly greater levels of carcass and non-carcass fat depots observed in this same treatment group. The anabolic effects of testosterone per se have been well documented (Butterfield 1988).

The suppression of stress-induced circulating cortisol concentrations by active immunization against ACTH was expected to increase growth rate and

decrease carcass fatness (Wynn *et al.* 1994). Despite the fact that the animals were maintained at pasture, the quality of feed on offer and the close supervision of animals most likely minimized the impact of stress: thus the lambs may have only been exposed to cortisol levels within the normal physiological range. The higher concentrations in the control group at day 32 relative to the ACTH-immune groups may simply reflect the suppression of circulating concentrations induced through the acute stress of yarding and bleeding of animals. By day 90 animals had most likely adapted to the handling and bleeding regime, as is evident from the lower levels in the control group, and so the divergence in cortisol status between control and ACTH-immune animals was not as evident. The observation of decreased plasma cortisol status over time with repeated yarding and handling of sheep has also been reported by Fell *et al.* (1991). The high variation in ACTH antibody titres is consistent with our previous observations, although it is interesting to note that this level of variation was not as evident in the GnRH antibody titres, suggesting that the latter antigen/adjuvant system was more effective.

In this study, the efficacy of GnRH immunization was altered by changing the HPA axis function through prior ACTH immunization. The biological activities of the HPA and the hypothalamic-pituitary-gonadal (HPG) axis are closely integrated. A previous study showed that the acute stress of repeated blood sampling of ewes resulted in changes to gonadotrophin levels and higher ovulation rates (Adams *et al.* 1993).

Therefore, in conclusion an alternative experimental model to ACTH immunization may be required to further investigate such links between the HPA and HPG axes in livestock production.

The authors acknowledge the skilled technical assistance of S. Pattison, E. Holland and M. O'Connor. The research was supported by a grant from the Meat Research Corporation.

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