

Humoral Immune Response to Equine Chorionic Gonadotropin in Ewes: Association with Major Histocompatibility Complex and Interference with Subsequent Fertility¹

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ABSTRACT

In dairy ewes, the use of eCG as a convenient hormone for the induction of ovulation is necessary for out-of-season breeding and artificial insemination (AI). In this report we show the presence of anti-eCG antibodies in plasma of treated ewes. The major histocompatibility complex (MHC) was involved in the individual variability of the humoral immune responses to eCG. We found significant associations between the anti-eCG response phenotype and some MHC class II alleles. The low immune response phenotype was associated with one MHC class II allele only in Lacaune ewes, and the high immune response phenotype was associated with one MHC class II allele both in Manech and in Lacaune ewes. In herds, the impact of residual anti-eCG antibodies on subsequent fertility after AI seems minimal because of an indirect elimination of high-responder ewes from AI breeding. Therefore, the true magnitude of the association between residual anti-eCG antibody concentration and fertility has been underestimated. An additional experiment without any high-responder female elimination showed a significant correlation between high residual antibody concentrations and lower lambing rate after AI at a fixed time, possibly because of a delayed preovulatory LH surge. The results suggest that anti-eCG antibody concentration is one risk factor for infertility after AI.

INTRODUCTION

Equine CG, a heavily glycosylated (45% w:w) glycoprotein hormone [1], is used for the induction of ovulation [2] for out-of-season breeding and artificial insemination (AI) in various species. In ewes, eCG exhibits a prolonged plasma half-life (up to 60 h [3]) mainly due to the terminal sialic acid residues of its *N*- and *O*-linked saccharide chains [4, 5]. This unique gonadotropin exhibits pronounced FSH activity in addition to its LH activity in species other than the horse [6–8]. These characteristics make eCG a convenient exogenous hormone for induction and synchronization of estrus and ovulation. In ewes, this hormonal treatment consists of a progestogen, flurogestone, delivered by vaginal sponge, followed by a 400–600-IU eCG injection [9].

The primary structure of eCG shares only 79% and 59% homology with the ovine pituitary LH and FSH, respectively [10]. Therefore, its heterologous origin, its high molecular weight (45 kDa) [6], and its high level of glycosyl-

ation render eCG potentially immunogenic. Recently, we demonstrated that the humoral immune responses against eCG were highly variable among individual goats [11]. A significant correlation was found between the anti-eCG antibody response and the major histocompatibility complex (MHC) class II alleles. Moreover, goats with high anti-eCG antibody concentration at the time of eCG injection (> 2.5 µg/ml) exhibited a much lower fertility after AI at a fixed time than did other females (41.3% vs. 66.7%), probably because of the delay observed both in the onset of estrus behavior and in the preovulatory LH surge.

In ewes, the presence of eCG-binding immunoglobulins after repeated eCG treatments is controversial. Some authors have reported that repeated eCG injections induced no antibody production [12, 13], while others reported that high plasma ¹²⁵I-eCG binding had negative effects on subsequent fertility [14]. Thus, the existence of a humoral immune response after eCG treatment in ewes, and of immune interference with subsequent fertility, remains unclear.

Here, we demonstrate the presence of anti-eCG antibodies in the plasma of treated ewes and analyze the kinetics of their secretion. The variability of these anti-eCG humoral immune responses corresponded to the MHC class II genotypes of females. In addition, a large-scale study in dairy ewes (Manech and Lacaune breeds) showed a correlation between anti-eCG antibody concentration and the subsequent fertility after AI.

MATERIALS AND METHODS

Anti-eCG Antibody Purification

Antibodies of preimmune and immune plasmas from nontreated (*n* = 2) or eCG-treated ewes (*n* = 2) were affinity purified on HiTrap protein G columns (Pharmacia, Uppsala, Sweden) that exhibit a high binding capacity for ovine immunoglobulins G (IgG) [15]. Briefly, 1.5 ml of plasma previously dialyzed against 20 mM sodium phosphate, pH 7, was loaded onto a HiTrap protein G column, and the wash-through (containing the IgM fraction) was collected. Bound IgGs were eluted using 0.1 M glycine buffer, pH 2.7. The collected fractions, immediately neutralized with 1 M Tris pH 9, were pooled and dialyzed overnight at 4°C against PBS, pH 7.4. Total IgG was concentrated to 8 mg/ml as determined by the method of Bradford [16]. The purified antibodies were then stored at –20°C until use.

Anti-eCG Humoral Immune Response

Fifty-four Lacaune ewes (1.5–5.5 yr of age; 20 without previous eCG treatment and 34 with one to three previous

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eCG treatments) from a farm in the Roquefort region were injected i.m. with a single dose of 500 IU eCG (Syncro-part, batch 13054A1; Sanofi Santé Nutrition Animale [SSNA], Libourne, France). Females were treated with eCG once a year according to manufacturer instructions. Jugular blood samples were collected into 5-ml heparinized tubes just prior to the injection of eCG and then twice weekly over 8 wk. The concentrations of anti-eCG antibodies in plasma were measured using a specific quantitative ELISA as described by Roy et al. [11]. Briefly, microtiter plates (CML, Angers, France) were coated with 250 ng (2.5 IU) of eCG (batch 13054A1; SSNA) in 100 μ l PBS, pH 7.4, overnight at room temperature. After blocking with 1% BSA (Organon, Boxtel, Holland) in PBS containing 0.1% Tween 20 (PBST), plasma samples and standards were distributed (100 μ l/well) in duplicate and incubated for 90 min at 37°C. Plasma samples were initially diluted from 1:50 to 1:200 in PBST containing 5% normal rabbit serum (PBST 5%R). Standard anti-eCG polyclonal ovine IgG previously purified [11] was serially diluted from 1000 to 7.5 ng/ml in PBST 5%R supplemented with 0.5% to 2% ovine plasma known to be free of anti-eCG antibodies. The peroxidase-conjugated rabbit anti-goat IgG polyclonal antibody (Jackson Labs., West Grove, PA) was then diluted to 1:5000 and incubated for 1 h at 37°C (100 μ l/well). Cross-reaction of the anti-goat IgG peroxidase-conjugated antibody with both goat and sheep IgG had been verified previously [11]. For isotype determinations, after incubation with ewe plasmas as described above, 100 μ l of the appropriate dilutions of mouse monoclonal antibodies against ovine IgG₁, IgG₂ [17], IgA, and IgM [18] were added (ascitic fluids kindly provided by Dr. K.J. Beh, CSIRO, Division of Animal Health, McMaster Laboratory, Glebe, Australia). After a 90-min incubation at 37°C, monoclonal antibody binding was detected with peroxidase-conjugated goat anti-mouse IgG (H and L chains; Dako, Glostrup, Denmark) diluted 1:5000 in PBST and incubated for 90 min at 37°C. In all cases, the peroxidase activity was detected with 100 μ l/well of a substrate mixture containing 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid; Sigma Chemical Co., St Louis, MO) at 0.04 M in 0.05 M citrate phosphate buffer, pH 4, and 0.01% H₂O₂. After incubation for 30 min at room temperature, the absorbance at 405 nm was measured with an automated microtiter plate reader Multiskan MK II (Labsystems, Helsinki, Finland). The eCG-specific IgG antibody concentration present in plasma was calculated from the linear part of the standard curve. Correction was made for plasma dilution, and antibody concentration was expressed as μ g/ml of plasma. Using pooled plasma from eCG-treated ewes as a positive control, intra- (n = 6) and interassay (n = 10) coefficients of variation were 6.0% and 11.0%, respectively.

Relationship between Anti-eCG Antibody Concentration and Fertility in Ewes

Animals and treatments. Manech and Lacaune ewes from different herds related to AI centers located in the South of France received a hormonal treatment (Syncro-part; SSNA) for synchronization and induction of estrus and ovulation for out-of-season breeding. Briefly, the females were treated for 14 days with a progestogen (vaginal sponge impregnated with 30–40 mg flurogestone acetate). At the end of the progestogen treatment, they were injected

i.m. with 500 IU eCG and inseminated with 400×10^6 fresh spermatozoa 55 ± 1 h later.

Experiment 1. The first experiment was performed in 1995 with 299 Manech ewes (three herds, 1.5–7.5 yr of age) and 309 Lacaune ewes (four herds, 1.5–7.5 yr of age). Under these field conditions, the distribution of females corresponded to five age groups of heterogeneous size. All females were treated and inseminated as described above. Blood samples were collected before eCG treatment (Day 0) and 10 and 25 days afterward (Day 10 and Day 25) in 1995. Plasma samples were stored at -20°C until measurement of anti-eCG antibody concentration by ELISA.

Experiment 2. This experiment was performed in 1996 with 542 Manech ewes (seven herds, 1.5–10.5 yr of age) and 719 Lacaune ewes (seven herds, 1.5–7.5 yr of age). Procedures were strictly identical to those of experiment 1, except that blood samples were collected only at Day 0.

Experiment 3. This experiment was performed in 1996 with 905 Lacaune ewes (ten herds, 1.5–8.5 yr of age), and females were treated and inseminated as described above. In contrast to procedures for experiments 1 and 2, these females were distributed into five identically sized groups by age (1.5, 2.5, 3.5, 4.5, and ≥ 5.5 yr). Blood samples were collected only before eCG treatment (Day 0). Plasma samples were stored at -20°C until measurement of residual anti-eCG antibody concentration by ELISA.

Among ewes used in experiment 1 (1995), some of the females were subjected to a new eCG treatment in 1996 either in experiment 2 or in experiment 3. Thus residual anti-eCG antibody concentrations obtained in 1996 could be compared with those observed the year before, in 1995, for 65 Manech and 85 Lacaune ewes.

Timing of the preovulatory LH surge was determined in two flocks of Lacaune ewes (n = 172) and two flocks of Manech ewes (n = 119). Blood samples were collected at 32, 36, 44, 52, and 55 h after eCG injection. Plasma samples were stored at -20°C until measurement of LH by ELISA (Reprokit, SSNA) [19].

In each ewe, the following parameters were recorded: herd; breed; age; number of previous eCG treatments; dairy production (only for Manech ewes); number of previous lambings; number of lambs at previous lambing; time interval between AI and previous lambing; anti-eCG antibody concentration at Day 0, Day 10, and Day 25 in experiment 1 and only at Day 0 in experiments 2 and 3; and fertility and prolificacy after AI. For the 719 Lacaune ewes in experiment 2, the number of previous lambings was unknown. For 179 of 905 Lacaune ewes (two herds) in experiment 3, the numbers of previous eCG treatments, previous lambings, and the time intervals between AI and the preceding lambing were missing. In order to compare these fertility results with previous findings [11, 14], females were distributed (60%, 20%, and 20%) into three classes according to anti-eCG antibody concentrations; 60% of females had the lowest antibody concentration and 20% the highest.

Microsatellite Analysis of MHC Polymorphism

Animals. Ewes used in experiment 1 were sorted according to their anti-eCG antibody concentration measured at the peak of the humoral immune response (Day 10). Only ewes exhibiting either low (< 7 μ g/ml) or high (> 15 μ g/ml) immune response phenotypes were selected for microsatellite analysis. This analysis was thus performed on 135 Manech ewes with low (n = 33) and high (n = 102) phe-

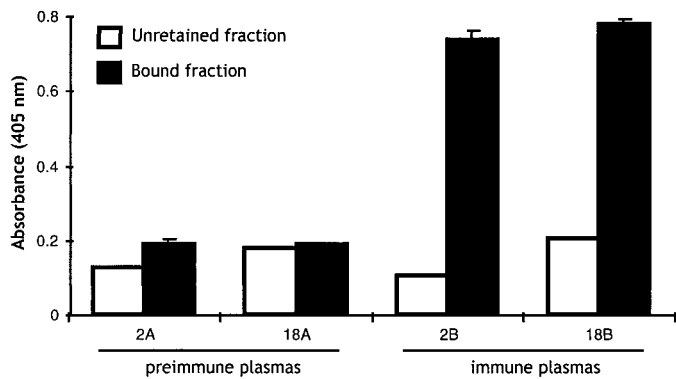


FIG. 1. Preimmune and immune plasmas, from two ewes injected i.m. with 500 IU eCG, fractionated by affinity chromatography with protein G. Ovine IgG was retained with a high binding capacity on protein G [15]. Absorbance values (mean \pm SEM) of unretained and eluted fractions were obtained by ELISA on eCG-coated plates.

notypes and on 107 Lacaune ewes with low ($n = 62$) and high ($n = 45$) phenotypes.

DNA extraction. Genomic DNA was prepared from the white blood cells by a fast and simple procedure (personal communication with Amigues Y, Labogena, Jouy en Josas, France). Briefly, venous blood was collected into vacutainer tubes containing EDTA. In 96-well, round-bottomed microtiter plates (Nunc, Roskilde, Denmark), 60 μ l of blood was distributed per well. After adding 150 μ l of NB buffer (10 mM NaCl, 10 mM EDTA, pH 7), the plates were centrifuged at $1500 \times g$ for 8 min. The supernatant was discarded, and the white cells were washed with 200 μ l of NB and centrifuged twice. After the last centrifugation, the pellet was homogenized in 200 mM NaOH (10 μ l) and heated at 65°C for 30 min. The mixture was neutralized with 100 mM Tris-HCl (10 μ l; pH 7) and diluted with 100 μ l H₂O. DNA was stored at 4°C or -20°C.

Polymerase chain reaction (PCR) amplification. OLADRB and BM1258, microsatellites located 13.2 cM (centimorgans) apart on ovine chromosome 20, inside and outside the MHC region, respectively, were amplified using the previously described primers [20, 21]. The PCR amplification was carried out in a Biometra (Whatman Biometra, Göttingen, Germany) thermocycler: 100 ng of genomic DNA and 0.2 μ M of each primer (one 5' end labeled with fluorescent dyes: Hex and 6-Fam for OLADRB and BM1258, respectively) were incubated for 2 min at 94°C. A mixture containing 1.6 mM MgCl₂, 67 mM Tris-HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 150 μ M of each dNTP, and 1.2 U of *Taq* DNA polymerase (Eurobio, Les Ulis, France) was added at 60°C according to the hot-start technique of Erlich et al. [22]. This step was followed by 35 cycles consisting of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and finally 72°C for 7 min. Then, 1.5 μ l of each PCR product was denatured for 2 min at 94°C after addition of 3.5 μ l of Tamra-500 internal standard (Perkin Elmer, Foster City, CA) diluted in formamide dye solution. Finally, 1.5 μ l of the denatured mixture was separated on a 5% denaturing polyacrylamide gel (SMC Bioproducts, Rockland, ME) using an ABI 377 automated sequencer (Perkin Elmer), and amplified DNA fragments were sized using the Genescan Analysis software program (Version 2.0; Perkin Elmer).

Statistical Analysis

Values are presented as means \pm SEM. Allele frequencies were determined by direct counting, and frequency dis-

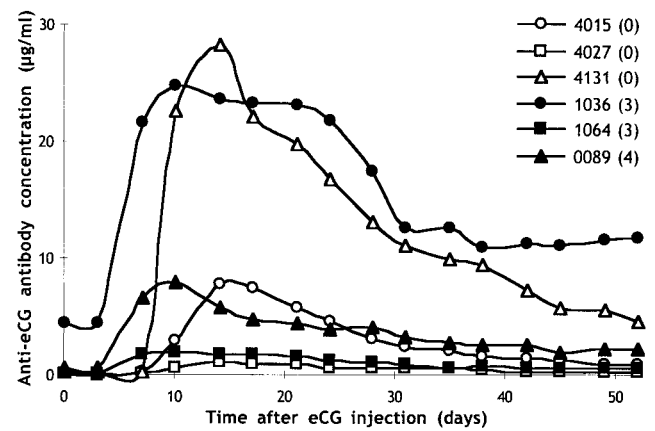


FIG. 2. Evolution of anti-eCG humoral immune responses (HIR) in Lacaune ewes injected i.m. with 500 IU of eCG at Day 0. HIR kinetics in six ewes were considered as representative of the entire group ($n = 54$). The number of previous eCG treatments is indicated in brackets. Anti-eCG antibody concentrations in plasma samples were determined by ELISA. Each point is the mean of duplicate determinations.

tributions for high and low phenotypes were compared using chi-square analysis for the size of classes > 5 . For anti-eCG antibody concentration at Day 0, Day 10, and Day 25, analysis of the distribution and searches for variation factors were carried out by multiple linear regression. Differences in anti-eCG antibody concentrations for each parameter analyzed according to several classes were determined by Student's *t*-test or ANOVA. The percentages of fertility were compared using chi-square analysis. To verify the effect of each main parameter on any other parameter, a multivariate analysis was performed and the fertility analysis was carried out using logistic regression. Analyzed parameters included herd, age, number of previous eCG treatments, dairy production, number of previous lambings, time interval between AI and the preceding lambing, and anti-eCG antibody concentration before (Day 0) and after (Day 10, Day 25) treatment. All the interactions between the various parameters were tested. Differences were considered significant for *p* values of 0.05 or less.

RESULTS

Demonstration of Anti-eCG Antibodies in Plasma of eCG-Treated Ewes

To confirm that the binding of eCG to plasma proteins was due to the presence of anti-eCG antibodies, preimmune and immune plasmas of two ewes were submitted to protein G affinity chromatography. No anti-eCG antibodies were detected in either the unretained or eluted fractions of preimmune plasmas ($n = 2$; Fig. 1). In contrast, ELISA data for eCG binding indicated that the eluted fractions from immune plasmas ($n = 2$) contained anti-eCG IgG antibodies (Fig. 1).

Humoral Immune Response Kinetics after One or More eCG Treatments

In ewes treated for the first time with eCG ($n = 20$), anti-eCG antibody concentration increased 10 days (Day 10) after eCG injection (Fig. 2). Maximum values were reached between Day 10 and Day 17; then a progressive decrease in anti-eCG antibody levels occurred over 2 mo. Ewes previously treated at least once with eCG ($n = 34$)

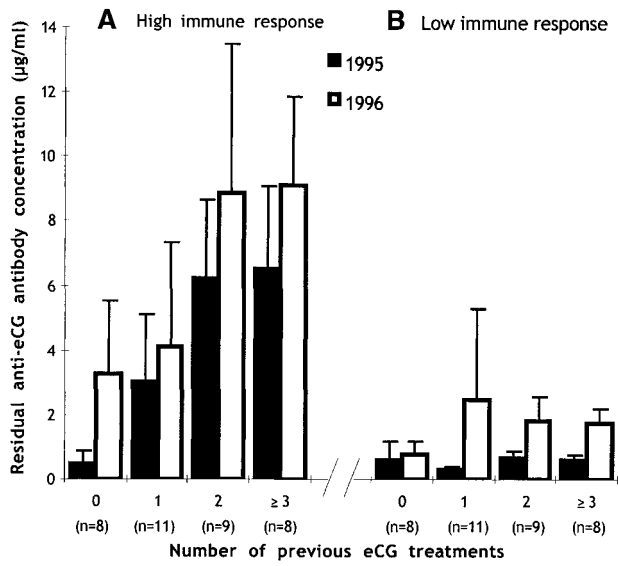


FIG. 3. Residual anti-eCG antibody concentrations (mean ± SEM) relative to the number of previous eCG treatments in the same females treated over two consecutive years (1995 and 1996) with eCG. Ewes were selected on the basis of their **A**) high (n = 36) or **B**) low (n = 36) humoral immune responses. The number of females is indicated in parentheses. Anti-eCG antibody concentrations were determined by ELISA.

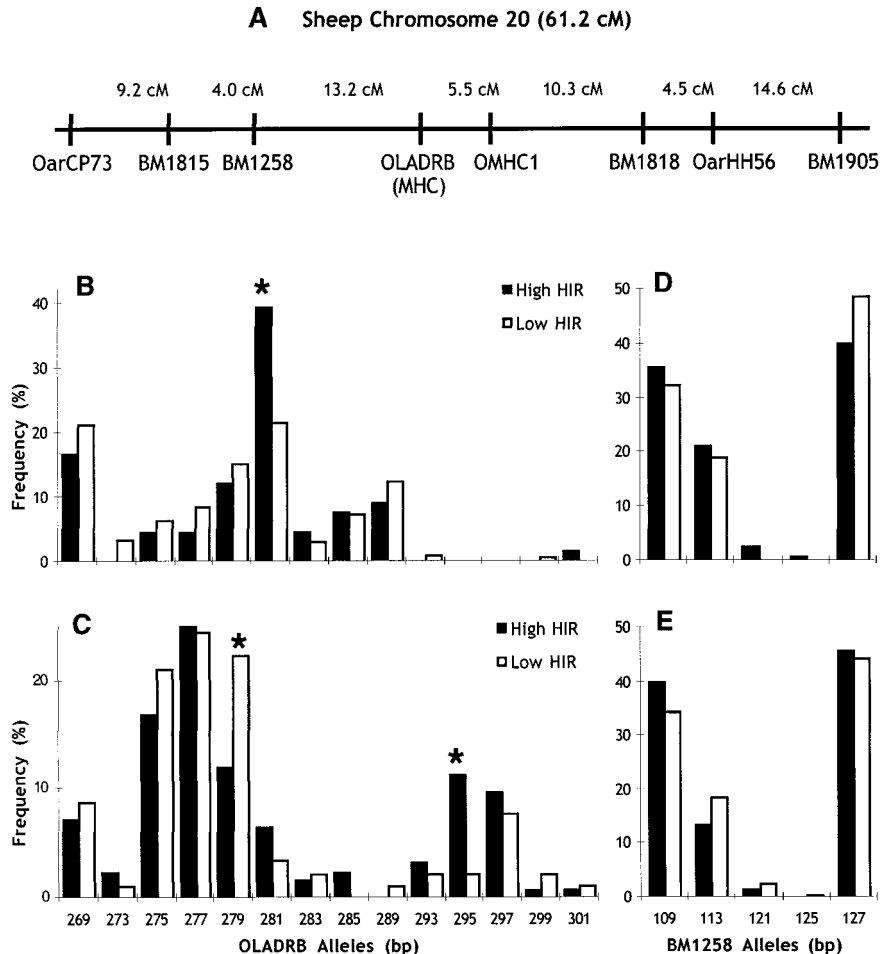
displayed similar humoral immune response kinetics, although their anti-eCG antibody concentrations increased earlier (at Day 7) and had a longer decreasing phase (Fig. 2). Primary and mainly secondary humoral responses were composed of anti-eCG IgG₁, with little or no IgG₂ and IgA antibodies (data not shown). We were not able to analyze IgM antibodies because of a constant high background in the ELISA. Anti-eCG antibodies, already present at the time of eCG injection and resulting from the previous immune response against eCG administered 1 yr earlier, were defined as residual antibodies.

Variable Humoral Immune Response among Individual Ewes after eCG Treatment

In spite of the similar kinetics of anti-eCG antibody secretion in ewes with either primary or secondary immune responses, each female in both groups differed markedly in its anti-eCG concentration (Fig. 2). Indeed, maximal anti-eCG antibody concentrations varied from 0.6 to 28.3 µg/ml in ewes treated for the first time and from 2.0 to 58.1 µg/ml in those treated several times.

Because of highly variable humoral immune responses, the evolution of the anti-eCG antibody response was evaluated in Lacaune and Manech ewes (n = 150) treated over two consecutive years with eCG. In the one fourth of the females (n = 36) with the highest humoral immune responses, the residual antibody concentration in plasma increased as a function of the number of previous eCG treatments (Fig. 3A). These ewes, systematically yielding high

FIG. 4. The OLADRB microsatellite is located on sheep chromosome 20 **A**) inside the MHC region. OLADRB allele frequency distributions are presented according to high and low anti-eCG humoral immune response (HIR) phenotypes in **B**) Manech (n = 135) and **C**) Lacaune (n = 107) ewes. BM1258, located outside the MHC region (**A**), was used as a microsatellite control, and allele frequency distributions are presented in **D**) Manech and **E**) Lacaune ewes. * Significant difference in allele frequency between high and low anti-eCG responders (p < 0.05).



antibody concentrations upon treatment, were designated high anti-eCG responders. Conversely, in the one fourth of ewes ($n = 36$) with the lowest humoral immune responses, the residual antibody concentration remained low independently of the number of eCG treatments (Fig. 3B), and these ewes remained poor responders upon subsequent treatment. Anti-eCG phenotype differences were particularly marked in ewes with three or more eCG treatments, since residual anti-eCG antibody concentrations in 1996 were $9.1 \pm 2.8 \mu\text{g/ml}$ and $1.7 \pm 0.5 \mu\text{g/ml}$ in high and low responders, respectively (Fig. 3).

Since different populations of ewes could be identified on the basis of their anti-eCG phenotypes, the genetic basis was explored. The MHC, which is involved in antigen presentation, was considered a potentially good candidate, and thus an association between MHC region polymorphism and variability in anti-eCG immune response was examined. The polymorphism in the DRB region of the MHC was analyzed by PCR amplification of a specific ovine OLADRB microsatellite (Fig. 4A) [20]. Manech and Lacaune ewes selected according to their anti-eCG antibody concentration at Day 10 in 1995 were distributed into groups of high or low anti-eCG responders. Twelve OLADRB alleles were identified in Manech and fourteen in Lacaune (Fig. 4, B and C). Their sizes ranged from 269 to 301 base pairs (bp). Manech and Lacaune ewes showed considerable variations in allele frequency distributions. In Manech (Fig. 4B), only the 281-bp allele was associated with the high immune response phenotype ($p < 0.005$). This frequent allele was encountered in 42% of the Manech ewes. In Lacaune (Fig. 4C), the 295-bp allele was associated with the high immune response phenotype ($p < 0.05$) whereas the 279-bp allele was associated with the low immune response phenotype ($p < 0.05$). These two alleles were encountered in 11% and 29% of Lacaune ewes, respectively. BM1258, a microsatellite located outside the MHC region at 13.2 cM from the OLADRB marker, was used as a control (Fig. 4A) [21]. Five BM1258 alleles were identified in Manech and Lacaune ewes, ranging in size from 109 to 127 bp (Fig. 4, D and E). In contrast to observations for OLADRB, and whatever the breed, BM1258 allele frequency distributions presented no difference between high and low anti-eCG responders.

Relationship between Anti-eCG Antibody Concentration and Fertility in Treated Ewes under Field Breeding Management

To analyze the effect of anti-eCG antibodies on subsequent fertility after AI at a fixed time, Manech and Lacaune ewes were examined in two large-scale experiments performed under field conditions (1995, $n = 608$; 1996, $n = 1261$). The overall fertility was lower in Manech than in Lacaune ewes (61.9% vs. 69.6% in experiment 1 and 58.3% vs. 64.0% in experiment 2, respectively; $p < 0.05$).

The effect of parameters on residual anti-eCG antibody concentration was tested in both breeds. In experiment 1 (1995), the number of previous eCG treatments, the age, and the interaction of the two had positive effects ($p < 0.001$). Moreover, anti-eCG antibody concentrations measured at Day 10 and Day 25 were correlated with the residual antibody concentration ($p < 0.001$). In experiment 2 (1996), residual antibody concentration increased with the number of previous eCG treatments and with the duration of the previous lambing-AI interval ($p < 0.001$).

In Figure 5, fertility results are presented in parallel with

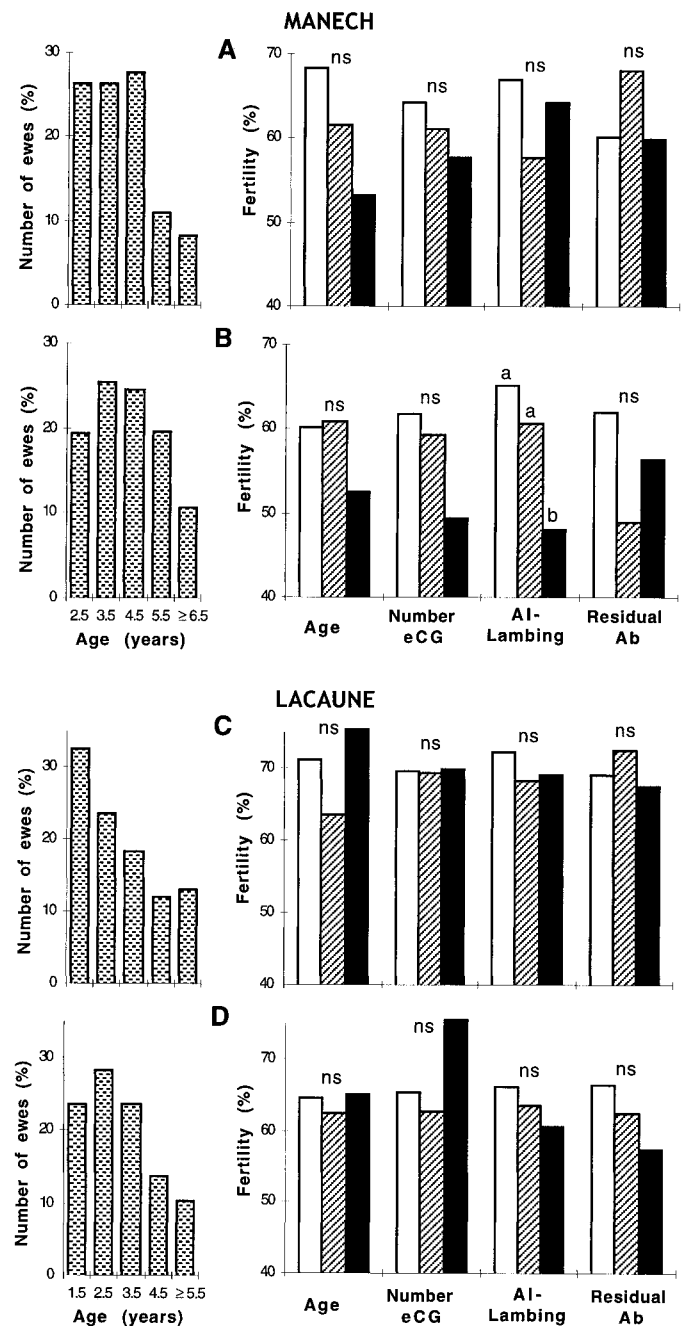
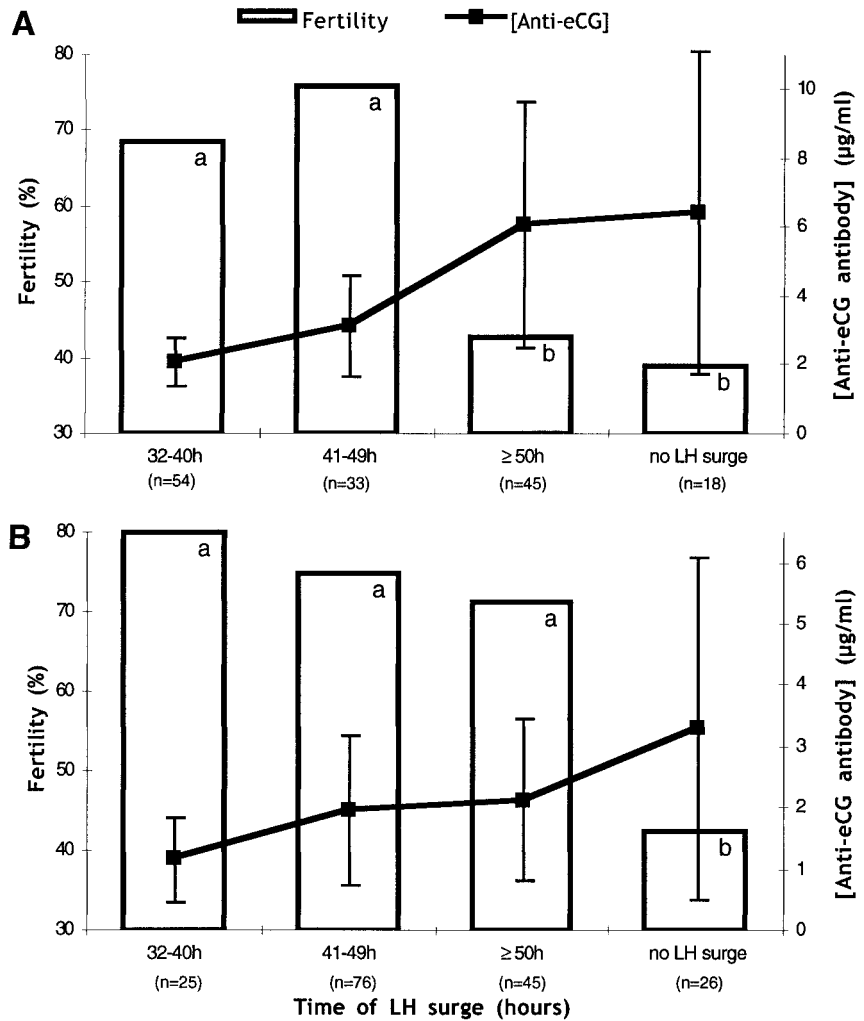


FIG. 5. Distribution of females under field conditions according to age in parallel with their fertility results over 2 yr in Manech (A: 1995 $n = 299$, B: 1996 $n = 542$) and Lacaune (C: 1995 $n = 309$; D: 1996 $n = 719$) ewes. Fertility results are presented according to three classes (open, striped, and solid bars) for the following variables: age of females (1.5–2.5, 3.5–4.5, and ≥ 5.5 yr), number of previous eCG treatments (0, 1–3, and ≥ 4), time interval between AI and the preceding lambing (> 210 , 210–180, and < 180 days), and distribution of females according to residual antibody concentration (60%, 20%, and 20%). Bars with different letters are significantly different ($p < 0.05$); ns, nonsignificant.

the age distribution of females. None of the described parameters had a significant effect on fertility, except that Manech females inseminated less than 180 days after previous lambing (in 1996) demonstrated a decrease in fertility rate ($p < 0.05$; Fig. 5B). In all experimental groups, prolificacies were not significantly different whatever the anti-eCG antibody concentrations (data not shown).

FIG. 6. Fertility and residual anti-eCG antibody concentration (mean \pm SEM) according to the time of LH surge after the end of the progestogen treatment in **A**) Lacaune ($n = 172$) and **B**) Manech ($n = 119$) breeds. The number of females is indicated in parentheses. Ewes were inseminated 55 ± 1 h after the end of the progestogen treatment. Bars with different letters are significantly different ($p < 0.05$).



A multivariate analysis of fertility was also performed to verify the effect of each main parameter on any other parameter. In Manech, this analysis demonstrated significant effects of herd and of the interaction herd \times residual antibody concentration in experiment 1 (1995). Herd, residual antibody concentration, and age were significant parameters in experiment 2 (1996). In Lacaune, herd was the only significant parameter related to fertility in 1996. In both breeds, anti-eCG antibody concentrations after treatment (Day 10 and Day 25) never appeared as significant parameters.

In addition, we assessed the residual antibody effect on the time of occurrence of the preovulatory LH surge in Manech ($n = 119$) and Lacaune ($n = 172$) breeds (Fig. 6). In both breeds, 15% of females showed no LH surge during the observation period. Among Manech ewes with an overall fertility of 56.5%, fertility dropped to under 45% in ewes with a delayed (> 50 h after sponge removal) or absent LH surge ($p < 0.05$; Fig. 6A). These females tended to have higher residual anti-eCG antibody concentrations, but the differences were not significant ($p = 0.15$). In Lacaune ewes with an overall fertility of 67.1%, fertility remained unchanged (71.1%) even in females with a late LH surge (Fig. 6B). Females with no LH surge exhibited a reduced fertility (42.3% vs. 75%; $p < 0.05$). These females tended to have higher residual anti-eCG antibody concentrations, but the differences were not significant ($p = 0.09$).

Interference of Anti-eCG Antibody with Fertility in Treated Ewes Out of Field Breeding Management

As demonstrated above, under field conditions, no negative effect of high residual antibody concentrations on fertility could be demonstrated in both breeds. However, in the Manech herd exhibiting the lowest fertility in 1995 (49%, $n = 104$), reduced lambing rate was correlated with high residual antibody concentration ($p < 0.05$). In fact, females with antibody concentration higher than $2.1 \mu\text{g/ml}$ ($n = 21$) exhibited 24% fertility (data not shown). Interestingly, we observed over experiments 1 and 2 that the distribution of residual antibody concentration increased in parallel with the mean number of eCG treatments in Manech and Lacaune ewes until 5.5 yr of age but, surprisingly, decreased in females older than 5.5 yr (Fig. 7). Therefore, it seems that under field conditions, old females selected for AI were only those with low residual anti-eCG antibody concentrations. In contrast, old females with high anti-eCG antibody concentrations had probably been culled from AI breeding because of their low fertility during the previous year. Accordingly, we assumed that high antibody concentration had a negative effect on subsequent fertility but may be hidden by breeding management conditions.

To test our hypothesis, Lacaune ewes were distributed either as in field ($n = 719$; heterogeneous classes of age) or in equilibrated distributions ($n = 905$; equal classes of



FIG. 7. Distribution of residual antibody concentrations (bars) in parallel with the mean of eCG treatments (open squares) as a function of age in Manech (1995, $n = 299$; 1996, $n = 542$) and Lacaune (1995, $n = 309$; 1996, $n = 719$) ewes over two consecutive years.

age) (Fig. 8). As previously observed, females in field distribution older than 5.5 yr had only low residual anti-eCG antibody concentrations ($\leq 10 \mu\text{g/ml}$; Fig. 8A), whereas females in equilibrated distribution older than 5.5 yr had much higher residual antibody concentrations ($\leq 30 \mu\text{g/ml}$; Fig. 8B). Under field conditions, none of the described parameters had a significant effect on fertility in Lacaune ewes (Fig. 9A), and herd was the only significant parameter in the multivariate analysis of fertility. In contrast, when females were distributed in classes of similar ages, fertility results were strikingly different (Fig. 9B). In fact, lambing rate decreased with 1) the age of ewes ($p < 0.001$), 2) the number of previous eCG treatments ($p < 0.005$), and 3) the time interval between previous lambing and AI ($p < 0.001$). With the residual antibody concentration parameter, a lower fertility rate (50.8%, $p < 0.01$) was also significant in females displaying high antibody concentration. Considering every parameter, the multivariate analysis of the fertility of ewes in equilibrated distribution confirmed that fertility decreased with age ($p < 0.001$), previous lambing-AI time interval ($p = 0.03$), and residual anti-eCG antibody concentration ($p = 0.045$).

DISCUSSION

The present study clearly demonstrates, by IgG affinity purification, the presence of anti-eCG antibodies in plasma of ewes treated with eCG. To date, the presence of eCG-binding immunoglobulins after eCG injection has been con-

troversial; some authors failed to detect antibody production after repeated eCG injections [12, 13], and others suggested that ^{125}I -eCG binding in plasma was probably due to the appearance of antibodies [14] but did not formally prove this. It is noteworthy that the heterologous origin, the high molecular mass (45 kDa) [6], and the high level of glycosylation (45% w:w) [1] of eCG render it potentially immunogenic. Even if nine antigenic sites mostly located on the α subunit of eCG have been characterized by immunomapping studies with monoclonal antibodies [23, 24], humoral immune response kinetics to eCG have been previously reported only in goats [11] and cats [25].

In ewes, kinetic studies showed that a single 500-IU eCG i.m. injection led to an immediate increase in anti-eCG antibody concentration with characteristics of primary or secondary humoral immune responses. Taken together, humoral immune responses in ewes and those previously reported for goats [11] indicate highly identical kinetics in anti-eCG antibody secretion. Analysis of anti-eCG antibody concentrations established a large variability in immunoglobulin secretion among sheep after eCG injection. Moreover, immunological responses to eCG appear variable between sheep breeds, since we observed lower anti-eCG antibody concentrations in Manech as compared to Lacaune ewes under similar eCG treatment. Moreover, maximal anti-eCG antibody concentrations appeared lower in ewes than those reported in goats [11].

In ewes, results obtained after two consecutive eCG

FIG. 8. Age distribution associated with the distribution of residual antibody concentrations (bars) in parallel with the mean of eCG treatments (open squares) as a function of age in Lacaune ewes in **A**) field ($n = 719$) and **B**) equilibrated ($n = 905$) distributions.

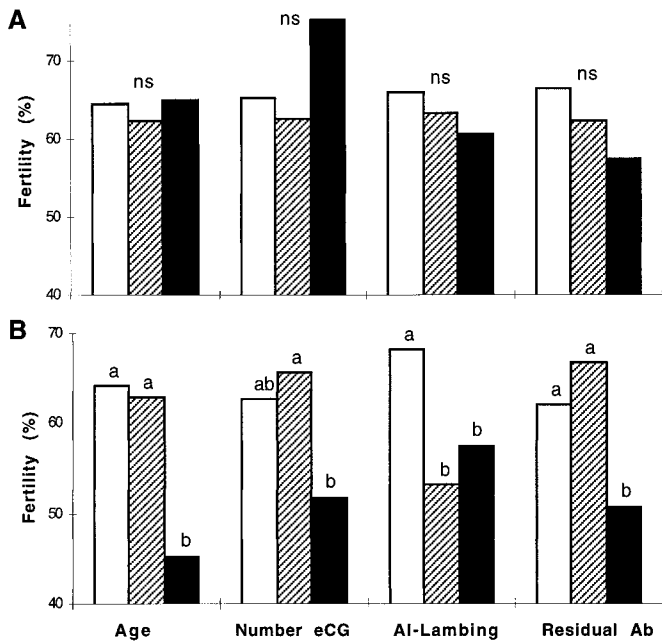
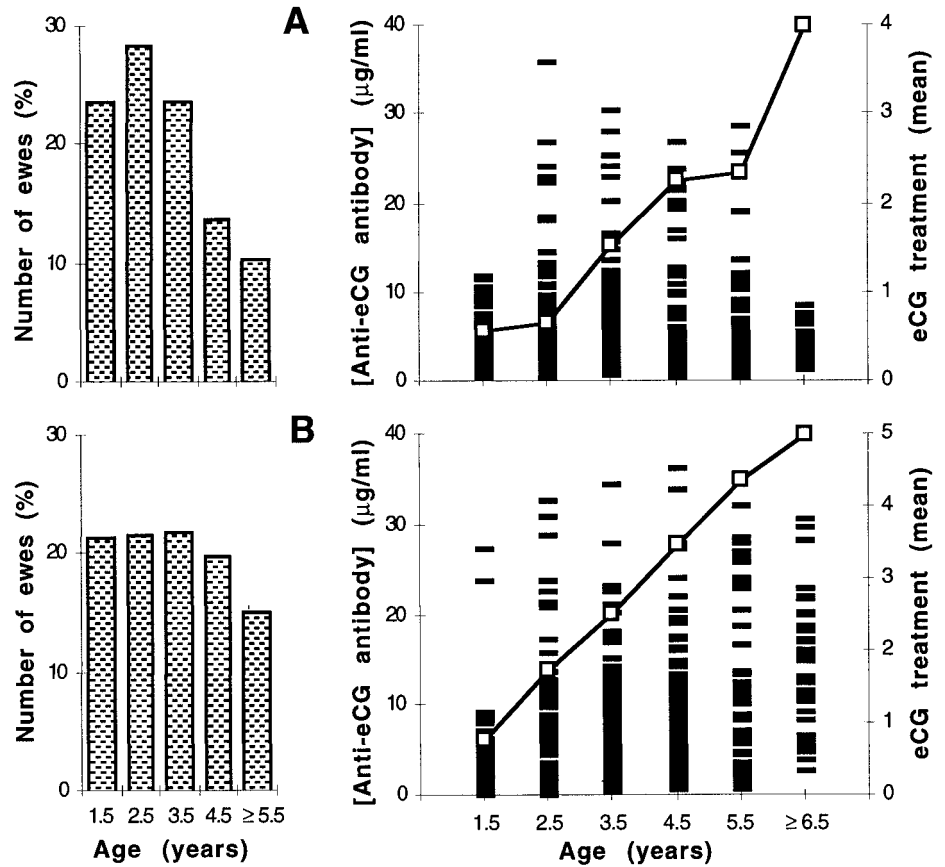


FIG. 9. Fertility in Lacaune ewes in **A**) field ($n = 719$) and **B**) equilibrated ($n = 905$) distributions. Fertility results are presented according to three classes (open, striped, and solid bars) for different variables: age of females (1.5–2.5, 3.5–4.5, and ≥ 5.5 yr), number of previous eCG treatments (0, 1–3, and ≥ 4), time interval between AI and the preceding lambing (> 210 , 210–180, and < 180 days), and distribution of females according to residual antibody concentration (60%, 20%, and 20%). Bars with different letters are significantly different ($p < 0.05$); ns, nonsignificant.

treatments indicate that the level of plasma antibody concentration is an inherent characteristic of each female, and they demonstrate the occurrence of different populations of ewes classified as low, medium, and high anti-eCG responders. Therefore, differences in immune responses between individuals, but also at interbreed or interspecies levels, might reflect genetic control of the anti-eCG antibody response. Therefore, some MHC genes, whose protein products control the ability to induce an immune response to exogenous proteins [26–28], must be involved. In particular, dimeric glycoproteins encoded by MHC class II genes present antigenic peptide to CD4+ T lymphocytes, which help B cells to produce appropriate immunoglobulins [29]. Class II genes are among the most polymorphic genes [30], and allelic differences in MHC molecules can modulate their ability to bind a large array of antigen-derived peptides.

Recently, we have found a significant association between anti-eCG immune response and three DRB alleles of MHC class II in goats [11] by use of the OLADRB microsatellite located inside the caprine MHC [31]. We examined such an association or so-called linkage disequilibrium in unrelated ewes with the OLADRB microsatellite located inside the MHC class II on sheep chromosome 20 [20]. BM1258, the closest marker located at 13.2 cM outside the MHC, was used as control [21]. The DRB region was found to be highly polymorphic in Manech and Lacaune sheep with allele sizes ranging from 269 to 301 bp, comparable to those reported in Merino ewes [20]. Considerable variations exist between Manech and Lacaune allele frequency distributions, but such breed differences have been previously reported elsewhere [32]. Here, we found significant associations between low anti-eCG response phenotype and

one MHC class II allele (279 bp) only in Lacaune ewes, and between high anti-eCG response phenotype and one MHC class II allele both in Manech (281 bp) and in Lacaune ewes (295 bp). We suggested previously that observed linkage disequilibrium between low-responder goats and the 280-bp allele might result from a selection pressure for high milk production and/or high fertility [11]. The association with the low response phenotype found only in Lacaune ewes also must be due to genetic improvement of females submitted to AI programs, practiced for longer than for Manech females. Moreover, we observed an indirect elimination of high-responder ewes from AI breeding, thus leading to a risk of selecting females with low humoral immune response not only against eCG but also against various antigens, including pathogens. Further studies should compare immune responses to eCG and other antigens.

Ovarian refractoriness or decreased fertility after repeated gonadotropin stimulations has been associated with the presence of gonadotropin-binding immunoglobulins in various species such as the rhesus monkey [33, 34], cat [25, 35], cow [36], and goat [11, 37]. This point has been less documented in sheep, and conflicting results concerning immunological interference with subsequent fertility have appeared in the literature. Some authors reported that failure of ewes to become pregnant after repeated eCG injections was not due to immunoneutralization of eCG [12, 13], whereas others reported significantly reduced fertility after AI in Lacaune ewes exhibiting high plasma ¹²⁵I-eCG binding compared to others (50.1%, n = 95 vs. 63.7%, n = 507) [14]. Therefore, we investigated the interference of anti-eCG antibodies with fertility in Manech and Lacaune ewes, the two main breeds used in dairy production in the South of France. The fertility analyses were performed separately on these two breeds because of differences between breeding management, region, and nutrition.

Under field conditions, a negative effect of high residual anti-eCG antibody concentrations on fertility was not evidenced in Manech and Lacaune females inseminated at a fixed time after treatment, except in the Manech herd with the lowest fertility. Nevertheless, a number of findings support the view that high residual anti-eCG antibody concentrations have negative effects on ewe reproduction. First, although chi-square and ANOVA tests did not indicate statistically significant differences under field conditions, our observations suggest 1) decreased fertility in ewes exhibiting high residual anti-eCG antibody concentration in 1996 and 2) higher residual anti-eCG antibody concentrations in females with an absent or late LH surge. Second, there was an unexpected bias in the distribution of Manech and Lacaune females, since high anti-eCG responders were absent among females older than 5.5 yr. In 1996, this bias seemed less important in Manech because 4 females out of 56 had antibody concentrations higher than 15 µg/ml. This bias demonstrates that old females with high anti-eCG antibody concentration are indirectly eliminated from AI breeding, probably because of their low fertility during the previous year.

Therefore, because of the observed bias in the distribution of females, the true magnitude of the association between residual anti-eCG antibody concentration and fertility was underestimated. An additional experiment was thus designed with Lacaune ewes distributed in identically sized age classes. In this situation, no female selection according to anti-eCG phenotype was observed, and fertility results were totally different, since a significant correlation be-

tween high residual antibody concentration and lower lambing rate was demonstrated. Unfortunately, LH surge detections were not feasible in these herds, and we could not confirm that high residual antibody concentrations were responsible for delayed ovulation. Our results indicate that anti-eCG antibody concentration is one risk factor of infertility after AI. However, other factors such as nutrition, stress, age, and breeding management might be also implicated in the reduced fertility after AI.

Compared to the situation in goats, interference of anti-eCG antibodies seems minimal in ewes for different reasons. First, the anti-eCG immune responses induced appeared lower in ewes than in goats [11], as previously observed with other antigens. Therefore, in females treated with the same dose of eCG, the immunoneutralization of the gonadotropin is greater in goats. Second, in goats, AI is performed with frozen-thawed semen whereas ewes are inseminated with fresh semen. Since fresh semen exhibits prolonged longevity compared to frozen semen, there is a less notable effect of the time of AI because the optimal window of insemination is thus enlarged in ewes. This explains the good fertility results observed in Lacaune females even with LH surges later than 50 h after the end of the progestogen treatment.

Whatever the anti-eCG antibody concentration, prolificacy was never affected, thus indicating that immunological interference did not provoke an abnormal ovarian response. In fact, residual anti-eCG immunoglobulins seem to interfere with injected eCG resulting in diminished ovarian stimulation and delayed follicular steroidogenesis, because only free eCG would bind to ovarian LH and FSH receptors. The affinity of antibodies induced by eCG injection in ewes, and the specificity of these antibodies, will have to be analyzed. However, it seems unlikely that anti-eCG antibodies interfere with endogenous LH and/or FSH, since cross-reactivity of eCG antibodies with endogenous gonadotropins were minimal in goats and ewes (unpublished results), monkeys [33], or cats [35]. Moreover, in several species, normal menstrual cyclicity and gestation, which are critically dependent on endogenous pituitary LH and FSH, were not impaired despite the presence of eCG-neutralizing immunoglobulins [33, 35]. Although monoclonal anti-eCG antibodies were shown to be inhibitors of eCG binding to LH receptors, and of LH and FSH bioactivities [23, 24], the mechanism of *in vivo* interference of polyclonal antibodies secreted by eCG-treated females on LH and FSH bioactivities of eCG is not yet known. Additional experiments are needed to obtain a thorough understanding of the immunological impact on the gonadotropin-induced reproductive function.

The results of the present study clearly demonstrate that repeated eCG treatments induce highly variable humoral immune responses among individual ewes; significant associations were found between anti-eCG response phenotype and some MHC class II alleles. Although anti-eCG antibodies have a negative effect on subsequent fertility after AI at a fixed time, the impact of residual anti-eCG antibodies on fertility in ewes is moderate under field conditions, mainly because of an indirect elimination of high-responder ewes from AI breeding.

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REFERENCES

- Christakos S, Bahl OP. Pregnant mare serum gonadotropin: purification and physicochemical, biological, and immunological characterization. *J Biol Chem* 1979; 254:4253–4261.
- Cole HH. Studies on reproduction with emphasis on gonadotropins, antigonadotropins and progonadotropins. *Biol Reprod* 1975; 12:194–211.
- McIntosh JE, Moor RM, Allen WR. Pregnant mare serum gonadotrophin: rate of clearance from the circulation of sheep. *J Reprod Fertil* 1975; 44:95–100.
- Martinuk SD, Manning AW, Black WD, Murphy BD. Effects of carbohydrates on the pharmacokinetics and biological activity of equine chorionic gonadotropin in vivo. *Biol Reprod* 1991; 45:598–604.
- Smith PL, Bousfield GR, Kumar S, Fiete D, Baenziger JU. Equine lutropin and chorionic gonadotropin bear oligosaccharides terminating with SO₄-4-GalNAc and Sia₂,3Gal, respectively. *J Biol Chem* 1993; 268:795–802.
- Combarnous Y, Salesse R, Garnier J. Physico-chemical properties of pregnant mare serum gonadotropin. *Biochim Biophys Acta* 1981; 667:267–276.
- Cole HH, Hart GH. The potency of blood serum of mares in progressive stages of pregnancy in effecting sexual maturity of the immature rat. *Am J Physiol* 1930; 93:57–68.
- Stewart F, Allen WR, Moor RM. Pregnant mare serum gonadotrophin: ratio of follicle-stimulating hormone and luteinizing hormone activities measured by radioreceptor assay. *J Endocrinol* 1976; 71:371–382.
- Gordon I. Artificial control of oestrus and ovulation. In: Gordon I (ed.), *Controlled Reproduction in Sheep and Goats*, vol 2. Cambridge: University Press; 1997: 86–115.
- Ward DN, Bousfield GR, Mar AO. Chemical reduction-reoxidation of the glycoprotein hormone disulfide bonds. In: Bellet D, Bidart JM (eds.), *Structure-Function Relationships of Gonadotropins*, vol 65. New York: Raven Press; 1989: 1–19.
- Roy F, Maurel MC, Combes B, Vaiman V, Cribiu EP, Lantier L, Pobel T, Delétang F, Combarnous Y, Guillou F. The negative effect of repeated equine chorionic gonadotropin treatment on subsequent fertility in alpine goats is due to a humoral immune response involving the major histocompatibility complex. *Biol Reprod* 1999; (in press).
- Gherardi PB, Martin GB. The effect of multiple injections of PMSG on the ovarian activity of Merino ewes. *Proc Aust Soc Anim Prod* 1978; 12:260.
- Diekman MA, Neary MK, Kelly GR. Repeated injections of pregnant mare serum gonadotrophin (PMSG) failed to induce antibody production in fall-lambing ewes. *J Anim Sci* 1995; 73(suppl 1): 51 (abstract 49).
- Bodin L, Drion PV, Remy B, Brice G, Cognié Y, Beckers JF. Anti-PMSG antibody levels in sheep subjected annually to oestrus synchronization. *Reprod Nutr Dev* 1997; 37:651–660.
- Åkerström B, Björck L. A physicochemical study of protein G, a molecule with unique immunoglobulin G-binding properties. *J Biol Chem* 1986; 261:10240–10247.
- Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem* 1976; 72:248–254.
- Beh KJ. Production and characterization of monoclonal antibodies specific for ewes IgG subclasses IgG₁ or IgG₂. *Vet Immunol Immunopathol* 1987; 14:187–196.
- Beh KJ. Monoclonal antibodies against ewes immunoglobulin light chain, IgM and IgA. *Vet Immunol Immunopathol* 1988; 18:19–27.
- Maurel MC. Development of an ELISA kit for the determination of LH on farm. In: Program of the 7th scientific meeting of the European Embryo Transfer Association; 1991; Cambridge. Abstract 176.
- Blattman AN, Beh KJ. Dinucleotide repeat polymorphism within the ovine major histocompatibility complex. *Anim Genet* 1992; 23:392.
- Crawford AM, Dodds KG, Ede AJ, Pierson CA, Montgomery GW, Garmonsway HG, Beattie AE, Davies K, Maddox JF, Kappes SW. An autosomal genetic linkage map of the sheep genome. *Genetics* 1995; 140:703–724.
- Erlich HA, Gelfand D, Sninsky JJ. Recent advances in the polymerase chain reaction. *Science* 1991; 252:1643–1651.
- Chopineau M, Maurel MC, Combarnous Y, Durand P. Topography of equine chorionic gonadotropin epitopes relative to the luteinizing hormone and follicle-stimulating hormone receptor interaction sites. *Mol Cell Endocrinol* 1993; 92:229–239.
- Maurel MC, Ban E, Bidart JM, Combarnous Y. Immunochemical study of equine chorionic gonadotropin (eCG/PMSG): antigenic determinants on alpha and beta subunits. *Biochim Biophys Acta* 1992; 1159:74–80.
- Swanson WF, Roth TL, Graham K, Horohov DW, Godke RA. Kinetics of the humoral immune response to multiple treatments with exogenous gonadotropins and relation to ovarian responsiveness in domestic cats. *Am J Vet Res* 1996; 57:302–307.
- Benacerraf B, McDevitt HO. Histocompatibility-linked immune response genes. *Science* 1972; 175:273–279.
- Schwartz RH. T-Lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu Rev Immunol* 1985; 3:237–261.
- Buus S, Sette A, Colon SM, Miles C, Grey HM. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* 1987; 235:1353–1358.
- Klein J, Figueroa F, Nagy ZA. Genetics of the major histocompatibility complex: the final act. *Annu Rev Immunol* 1983; 1:119–142.
- Bodmer JG, Marsh SGE, Albert E. Nomenclature for factors of the HLA system. *Immunol Today* 1990; 11:3–10.
- Vaiman D, Schibler L, Bourgeois F, Oustry A, Amigues Y, Cribiu EP. A genetic linkage map of the male ewes genome. *Genetics* 1996; 144:279–305.
- Moazami-Goudarzi K, Laloë D, Furet JP, Grosclaude F. Analysis of genetic relationships between 10 cattle breeds with 17 microsatellites. *Anim Genet* 1997; 28:338–345.
- Bavister BD, Dees C, Schultz RD. Refractoriness of rhesus monkeys to repeated ovarian stimulation by exogenous gonadotropins is caused by nonprecipitating antibodies. *Am J Reprod Immunol Microbiol* 1986; 11:11–16.
- Ottobre JS, Stouffer RL. Antibody production in rhesus monkeys following prolonged administration of human chorionic gonadotropin. *Fertil Steril* 1985; 43:122–128.
- Swanson WF, Horohov DW, Godke RA. Production of exogenous gonadotrophin-neutralizing immunoglobulins in cats after repeated eCG-hCG treatment and relevance for assisted reproduction in felids. *J Reprod Fertil* 1995; 105:35–41.
- Jainudeen MR, Hafez ESE, Gollnick PD, Moustafa LA. Antigonalotropins in the serum of cows following repeated therapeutic pregnant mare serum injections. *Am J Vet Res* 1966; 27:669–675.
- Baril G, Remy B, Leboeuf B, Beckers JF, Saumande J. Synchronization of estrus in ewes: the relationship between eCG binding in plasma, time of occurrence of estrus and fertility following artificial insemination. *Theriogenology* 1996; 45:1553–1559.