Immunosuppressive properties of follicular fluid from preovulatory horse follicles

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Summary. Fluid was aspirated from the preovulatory follicles of mares before and 12, 24 and 36 h after intravenous administration of hCG. Follicular fluid significantly ($P < 0.001$) reduced lymphocyte blastogenesis *in vitro* and, at a dilution of 1:100, fluid collected at 36 h after administration of hCG was significantly more suppressive ($P < 0.01$) than fluid collected before 36 h. Suppression of blastogenesis was reduced by extracting the follicular fluid with ether or by charcoal treatment ($P < 0.01$) or by heating at 56°C for 30 min ($P < 0.05$). Preincubation of lymphocytes with 2 of 5 follicular fluid samples expressed subsequent blastogenesis. Follicular fluid inhibited blastogenesis of T-cell growth factor (TCGF)-dependent Con A lymphoblasts ($P < 0.05$) and the degree of inhibition was related to time of addition of the TCGF and time of collection of the follicular fluid. These results indicate that preovulatory follicular fluid in the mare is increasingly suppressive to lymphocytes as time of ovulation approaches and that this immunosuppression is associated with an alteration of the response to lymphokine stimulation.

Keywords: mare; immunosuppression; follicular fluid

Introduction

Both the oocyte and fertilized ovum are potentially antigenic to the maternal immune system and may be susceptible to the mare's immune defence mechanisms. As the process of ovulation is believed to be an inflammatory response (Espey, 1980), it is important that the inflammatory reaction be curbed before it proceeds to an immune response. In several species, including the horse, antibodies to the zona pellucida have been detected and can prevent spermatozoa from attaching and penetrating ova (Ownby & Shivers, 1972; Glass & Hanson, 1974; Trounson *et al.*, 1980; Shivers & Liu, 1982). It has also been shown that antibodies directed against the zona pellucida can inhibit shedding of the zona by the embryo (Dudkiewicz *et al.*, 1975).

The preovulatory follicle in the mare ovulates when it reaches a mean diameter of 45 mm (Pierson & Ginther, 1985). At ovulation, the infundibulum surrounds the ovary to facilitate passage of the oocyte to the ampulla. This means that, in addition to the oocyte, approximately 45 ml follicular fluid theoretically will pass into the oviduct. It seems possible, therefore, that substances are normally present in follicular fluid which protect the oocyte and developing embryo by suppressing the maternal immune system. Ultrasonographic results suggest that much of the evacuated fluid does not collect in the ipsilateral oviduct (Townson & Ginther, 1989), but the oviduct in ovulating mares was not positively identified. Follicular fluid from women (Fahmi *et al.*, 1985) and cattle (Castilla *et al.*, 1988) suppresses mitogen-induced lymphocyte blastogenesis *in vitro* and in women this mechanism appears to operate via induction of suppressor cell activity (Castilla *et al.*, 1988). Suppressor/cytotoxic lymphocytes (T8) have also been detected in follicular fluid from women (Hill *et al.*, 1987).

The present study investigated the effect on mitogen-induced lymphocyte blastogenesis of plasma or of follicular fluid collected from preovulatory follicles of mares at different intervals after administration of hCG. T-cell growth factors (TCGF), which are mainly interleukin-2 (IL-2) (Oldham & Williams, 1984), are produced by activated T lymphocytes to induce clonal proliferation of T cells. By adding a source of TCGF to activated lymphocytes, it was possible to test whether follicular fluid interfered with TCGF-induced blastogenesis.

Materials and Methods

Animals and experimental design. Normally cycling, light breed horse mares aged between 3 and 16 years were used. Oestrus was detected by use of a teaser stallion. From the first day of oestrus, each mare's ovaries were examined by ultrasonography. On the day that the preovulatory follicle reached 35 mm, 2500 i.u. hCG (Butler Co., Columbus, OH, USA) were administered intravenously. Follicular fluid was aspirated either before administration of hCG or 12, 24 or 36 h later. Follicular fluid from 4 mares was aspirated at each time interval: 1 of the mares at 12 h, 1 at 36 h and 2 at 24 h had two preovulatory follicles of equivalent size. In these mares, fluid was aspirated from both follicles. At the time of aspiration, blood was collected from the jugular vein into a heparinized evacuated tube. Plasma was stored at -20°C .

Aspiration of follicular fluid. Follicular fluid was aspirated using a technique described previously in which, after appropriate sedation and analgesia of the mare, the ovary was held by a hand inserted into the abdomen via an incision in the cranial vagina and the fluid aspirated via a needle inserted through the flank (Hinrichs & Kenney, 1987). After collection, the follicular fluid was stored on ice until centrifuged at 2000 *g* for 10 min at 4°C . The supernatant was then stored in aliquants at -70°C . None of the samples contained significant red blood cell contamination.

Follicular fluid treatments. Follicular fluid collected from follicles 36 h after administration of hCG was subjected to various treatments: (1) 25 mg charcoal was added to 0.75 ml follicular fluid and incubated at room temperature for 30 min with frequent agitation. The mixture was then centrifuged at 2000 *g* for 1 h at 4°C and the supernatant removed and added to a lymphocyte blastogenesis assay. (2) The fluid was heated at 56°C for 30 min before being included in the assay. (3) The fluid (1 ml) was extracted twice with ethyl ether (15 ml). The extract was resuspended in 1 ml medium and both the extract and the follicular fluid were added to a lymphocyte blastogenesis assay. Blastogenesis results were compared with wells containing medium which had been subjected to the same treatments.

Lymphocyte blastogenesis assay. Lymphocytes were isolated from blood of a healthy mare and the assay performed as previously described (Watson, 1990). Follicular fluid (100 μl), undiluted and at dilutions of 1:20 and 1:100 or plasma collected at the time of aspiration (100 μl ; 1:20) was added to wells containing lymphocytes (1×10^5) and concanavalin A (Con A 50 μl , 20 $\mu\text{g}/\text{ml}$; Sigma Chemical Co, St Louis, MO, USA). Control wells were included in which medium was substituted for follicular fluid or for Con A. After 72 h incubation, 0.5 μCi [^3H]thymidine (sp. act. 6.7 Ci/mmol; New England Nuclear, Boston, MA, USA) was added to each well. The wells were harvested 6 h later. Each follicular fluid or plasma sample was added to wells in quadruplicate.

TCGF production by mitogen-treated cells. Peripheral blood mononuclear cells were isolated as previously described (Watson, 1990) and were cultured with 80 μg Con A/ml for 2 h as described by Oldham & Williams (1984). Control cultures were performed in the absence of Con A. Cells were washed twice before being incubated for an additional 24 h. The medium was then harvested by centrifugation at 2000 *g* for 10 min and the supernatant stored in aliquants at -70°C .

TCGF assay. TCGF-dependent cells were produced as previously described (Oldham & Williams, 1984) by culturing mononuclear cells with Con A (5 $\mu\text{g}/\text{ml}$) for 4 days. Cells were washed twice and resuspended at $2 \times 10^5/\text{ml}$ before being used in the assay. Serial 2-fold dilutions of the TCGF cell supernatants (100 μl) plus 100 μl medium were added to triplicate cultures of cells (50 μl). A 1:2 dilution of the TCGF supernatant was added to wells containing cells plus follicular fluid (100 μl). After a 24-h incubation, [^3H]thymidine (1 μCi) was added to each well and harvested after a further 18 h. Control wells were included in which medium replaced the TCGF supernatant. In another assay, TCGF supernatant was added 6 h after the start of the assay.

Induction of suppressor cells. Mononuclear cells were preincubated for 48 h at 37°C in an humidified atmosphere of 5% CO_2 :95% air with a 1:2 dilution of follicular fluid aspirated 36 h after hCG. Controls were included in which follicular fluid was replaced by complete medium (RPMI-1640 containing 10% fetal calf serum, 10^{-5} M-2-mercaptoethanol, 20 mM-Hepes, 2 mM-L-glutamine, 50 units penicillin/ml, 50 μg streptomycin/ml). The cells were then washed twice and resuspended at $2 \times 10^6/\text{ml}$. These cells (50 μl) were then added to wells containing freshly prepared autologous blood mononuclear cells (50 μl , $2 \times 10^6/\text{ml}$) and included in a lymphocyte blastogenesis assay.

Determination of cell viability. In each assay, wells were included in which [^3H]thymidine was replaced by complete medium. At the end of the assay, the viability of the cells was assessed by exclusion of trypan blue dye.

Statistical analyses. Degree of suppression of [^3H]thymidine incorporation was expressed as a percentage of c.p.m. in control wells. A one-way or repeated measures analysis of variance was performed to analyse effects of treatment.

When appropriate, means were compared using the least significant difference equation. Results were considered significant when $P < 0.05$.

Results

Follicular fluid undiluted and at a dilution of 1:20 significantly suppressed lymphocyte blastogenesis (Fig. 1). At a dilution of 1:100, only fluid collected 36 h after hCG was significantly immunosuppressive. Undiluted follicular fluid was equally suppressive at all collection times. At a 1:20 dilution, follicular fluid collected at 36 h was more suppressive ($P < 0.05$) than fluid collected at 12 h and tended ($P < 0.1$) to be more suppressive than fluid collected at 0 and 24 h. When diluted to 1:100, follicular fluid collected at 36 h was significantly ($P < 0.01$) more suppressive than fluid collected at other time intervals. Viabilities of cells suspended in undiluted follicular fluid were $76.3 \pm 5.4\%$ in 0 h, $92.0 \pm 3.7\%$ in 12 h, $90.0 \pm 1.5\%$ in 24 h, and $88.0 \pm 2.8\%$ in fluid collected 36 h after administration of hCG. Viability in medium was $86.7 \pm 2.9\%$. Addition of plasma to wells only slightly suppressed blastogenesis ($0.05 < P < 0.1$) compared with control wells (0 h, $73.1 \pm 2.9\%$; 12 h, $91.7 \pm 6.5\%$; 24 h, $87.1 \pm 6.1\%$; 36 h, $76.4 \pm 7.4\%$). Although ether extraction, charcoal stripping, and heat treatment reduced suppression of blastogenesis by follicular fluid ($21 \pm 3.9\%$, $P < 0.01$; $16.1 \pm 2.3\%$, $P < 0.01$; and $11.9 \pm 3.3\%$, $P < 0.05$; respectively), the fluid still significantly suppressed blastogenesis ($P < 0.001$). When cells were preincubated in follicular fluid for 48 h before assay (Table 1), 2 of the 5 follicular fluid samples collected 36 h after administration of hCG induced suppressor cell activity. Blastogenic response to TCGF was inhibited

Fig. 1. Effect of follicular fluid on Con A-induced lymphocyte blastogenesis. Follicular fluid collected 0 ($n = 4$), 12 ($n = 5$), 24 ($n = 6$), and 36 ($n = 5$) h after administration of hCG was added to wells undiluted and at dilutions of 1:20 and 1:100. Results are expressed as % of control wells which did not contain follicular fluid. Values with superscripts are significantly different from controls. a, $P < 0.01$; b, $P < 0.001$.

Fig. 2. Effect of follicular fluid on TCGF-induced lymphocyte blastogenesis. Follicular fluid was added to wells and supernatant containing TCGF was then added immediately or 6 h after the beginning of the assay. Results are expressed as % of control wells which did not contain follicular fluid. * $P < 0.05$; ** $P < 0.01$. Significant difference in immunosuppression between addition of TCGF at the start of the assay or 6 h later.

Table 1. Effect of preincubation of cells for 48 h with follicular fluid on subsequent blastogenesis

Preincubation medium	Incorporation of [^3H]thymidine (c.p.m.)
Follicular fluid 1	26 227 $\pm$ 639
Follicular fluid 2	27 022 $\pm$ 598
Follicular fluid 3	14 339 $\pm$ 1261 ^a
Follicular fluid 4	19 907 $\pm$ 1261
Follicular fluid 5	18 734 $\pm$ 1322 ^b
Medium	22 363 $\pm$ 1566

Values are the mean $\pm$ s.e.m. of 4 replicate wells.

Figures with superscripts are significantly different from control. a, $P < 0.001$; b, $P < 0.05$.

($P < 0.001$) after addition of follicular fluid (Fig. 2). When addition of TCGF was delayed by 6 h, immunosuppression was reduced in wells containing follicular fluid collected at 12, 24 and 36 h compared with wells in which TCGF was added at the start of assay.

Discussion

This study showed that fluid collected from preovulatory horse follicles suppressed lymphocyte blastogenesis, apparently via alteration of the IL-2 system. When hCG is administered to mares which have a follicle approximately 35 mm in diameter in their ovaries, ovulation normally occurs within 36–48 h (Duchamp *et al.*, 1987). The present results show that the degree of immunosuppression by follicular fluid increased as time of ovulation approached. Previous work with cattle (Fahmi *et al.*, 1985) showed that large follicles possessed more immunosuppressive activity than did small follicles and preovulatory follicles, but there appears to be no information on fluid from maturing preovulatory follicles in any species.

Follicular fluid is formed in part by transudation from serum (Edwards, 1974; Andersen *et al.*, 1976). Little immunosuppressive activity was present in plasma and no differences were detected between samples collected at different time intervals after administration of hCG. It therefore seems likely that, as in the cow (Fahmi *et al.*, 1985), the lymphocyte inhibitory factors are either locally produced or selectively transported from plasma into the follicles.

Measurements of lymphocyte viability revealed similar survival rates between cells cultured in follicular fluid and in control wells containing complete medium. The immunosuppressive effect was therefore not mediated by lymphocyte cytotoxicity. Follicular fluid collected from women suppressed the proliferative response of autologous lymphocytes by inducing or enhancing suppressor cell activity (Castilla *et al.*, 1988). Such activity was apparent in only 2 of 5 follicles when mare follicular fluid was preincubated with lymphocytes before their inclusion in a lymphocyte blastogenesis assay. Differences between individual cows in immunosuppressive factors in preovulatory follicular fluid have been reported by Fahmi *et al.* (1985) and these workers suggested that it was advisable to study samples from individual animals, rather than pooled samples, around the time of ovulation.

It appeared that follicular fluid interfered with TCGF induction of lymphocyte blastogenesis. When addition of TCGF was delayed by 6 h, suppression of lymphocyte blastogenesis was significantly decreased. This suggests that follicular fluid was not merely binding TCGF directly, making them unavailable for lymphocyte stimulation, or masking IL-2 receptors. The follicular fluid could have been acting to suppress protein synthesis within the lymphocytes, reducing IL-2 receptor expression and blastogenesis. If the action of follicular fluid was to decrease TCGF production, blastogenesis should not have been suppressed in the present experiment in the presence of a source of exogenous TCGF.

As the follicle of the mare matures towards ovulation, many hormonal changes occur within the follicular fluid (E. D. Watson & P. L. Sertich, unpublished). In particular, there are large increases in concentrations of progesterone, PGF, PGE-2 and 6-keto-PGF-1 α between 24 and 36 h after administration of the hCG. High concentrations of progesterone are known to suppress lymphocyte blastogenesis (Low & Hansen, 1988) and PGE-2 suppresses both mitogen- and IL-2-stimulated lymphocyte blastogenesis in cows, ewes (Low & Hansen, 1988), and mares (E. D. Watson, unpublished data). The present study shows that hormones in follicular fluid may be acting to suppress lymphocyte blastogenesis, thereby reducing the immune response of the mare to antigens from the oocyte and the embryo. Treatment of follicular fluid with charcoal, heat, and ether extraction of the fluid slightly reduced its suppressive effect on lymphocyte blastogenesis. Both charcoal treatment and ether extraction would remove prostaglandins and gonadal steroids from the fluid but not proteins. It therefore appears likely that the former substances are contributing to the immunosuppression. By contrast, Fahmi *et al.* (1985) found that the lymphocyte inhibitory factors present in bovine follicular fluid were heat-labile which ruled out the possibility that gonadal steroids were responsible. In our study, the high degree of immunosuppression still present after treatments indicates that the main inhibitory factor(s) remains unknown but may be a protein rather than steroid. In conclusion, follicular fluid from mares possess immunosuppressive properties which may function to inhibit an oviducal immune response to the zona pellucida.

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