

Mechanisms and physiological implications of leucocyte chemoattraction into periovulatory ovine follicles

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Leucotactic polypeptide was isolated from follicular-conditioned incubation media by ultra-filtration and reverse-phase high performance liquid chromatography. The bioactive fraction was subjected to amino acid analysis and shown to be abundant in glycine, proline and hydroxyproline. These amino acid residues are common to repetitive sequences of α collagens. Synthetic collagen-like peptides composed of repeating triplets of glycine, proline and hydroxyproline (GPH \times 3, GPH \times 9) were active in attracting white blood cells as measured *in vitro* using a linear under-agarose assay. Accumulation of leucocytes within extravascular spaces of the theca interna was induced by intrafollicular injection of GPH \times 3 or bacterial collagenase. Intrafollicular administration of affinity-purified GPH \times 3 antibodies during the immediate preovulatory period inhibited thecal extravasation of leucocytes, but did not negate follicular rupture. However, serum concentrations of progesterone were depressed in antibody-treated animals throughout the ensuing luteal phase. This luteal defect was counteracted by injecting leucocytes into the preovulatory follicle. It is concluded that periovulatory follicles of the sheep secrete collagen-like leucotactic peptides. Once drawn into the follicle, resident inflammatory cells are apparently involved in transforming it into a fully functional corpus luteum.

Introduction

It is becoming increasingly evident that white blood cells attracted from the circulatory system into the ovaries could be involved in the mechanics of follicular atresia, ovulation and luteal regression (Adashi, 1990; Mori, 1990; Coulam and Stern, 1991). However, there are few data concerning the chemical nature of leucocyte chemoattractants derived specifically from ovarian tissues, or whether occupant leucocytes serve an obligatory physiological function.

We previously isolated a low molecular weight substance secreted *in vitro* by periovulatory ovine follicles that attracts granulocytes and monocytes. Partial purification of this material indicated that it was a polypeptide rich in glycine (Murdoch and McCormick, 1989). As reported herein, a complete amino acid analysis of the composition of the putative chemoattractant demonstrated that it also contains relatively high amounts of proline and hydroxyproline. Because the main body chain of collagen consists of repeating sequences of glycine-X-Y, where X and Y are often proline or hydroxyproline (Harper, 1980), and since thecal collagen is degraded during the process of follicular rupture (Woessner *et al.*, 1989), we hypothesized that the leucocyte chemoattractant comprises fragments of collagen liberated in association with the dissolution of the connective tissue matrix of the wall of the ovulatory follicle. Additional experiments were carried out to test the efficacy of synthetic collagen-like peptides on the migration of sheep leucocytes *in vitro*, to evaluate the leucotactic properties of intrafollicular

injection of collagen-like peptide and collagenase, and to determine the effects of affinity-purified immunoglobulin generated against collagen-like peptide on follicular leucocyte migration and consequent ovarian functions *in vivo*.

Materials and Methods

Materials

Unless otherwise specified, reagents were purchased from Sigma Chemical Co., St Louis, MO.

Animal model

Mature Western-range ewes were penned with vasectomized rams daily and observed for oestrous behaviour. Day 0 was considered as the first day of oestrus. Luteal regression and the onset of the preovulatory surge of luteinizing hormone (LH) were synchronized by treating animals i.m. with 10 mg of prostaglandin $F_{2\alpha}$ (Lutalyse: The Upjohn Co., Kalamazoo, MI) on day 14 followed 36 h later by 5 μ g of an agonistic analogue of LH-releasing hormone (des-Ala⁶-Gly¹⁰ ethylamide; LHRH). The dominant follicle (6–8 mm in diameter) in the pair of ovaries will consistently ovulate about 24 h after administration of LHRH (Roberts *et al.*, 1985).

Surgery

Animals were not allowed access to food and water for 24 h before surgery. All surgical procedures were performed

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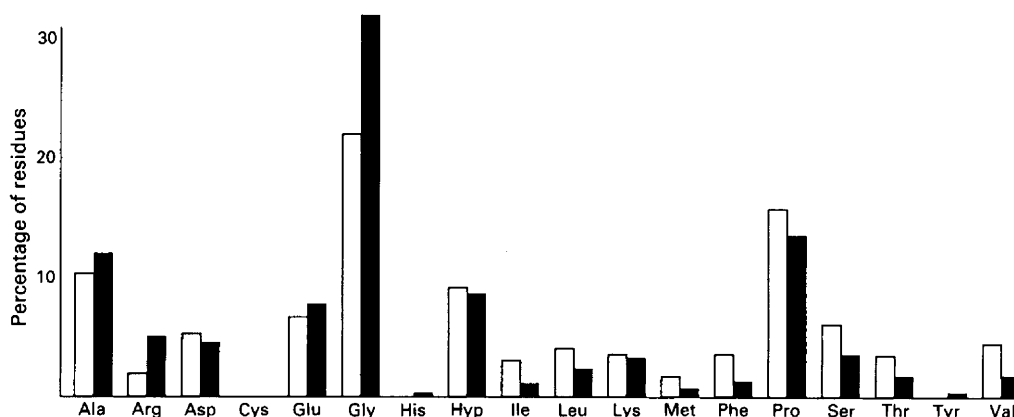


Fig. 1. Comparison of the amino acid composition of the bioactive HPLC fraction isolated from conditioned media of periovulatory follicles and bovine tendon collagen. (□) ovine follicular peptide; (■) bovine collagen.

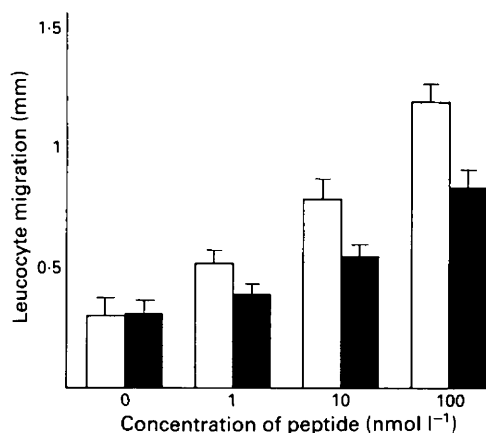


Fig. 2. Induction of leucocyte chemotaxis (leading front of migration under agarose) by synthetic collagen-like peptides (□) GPH x 3; (■) GPH x 9. Both peptides stimulated a linear increase ($P < 0.05$) in activity. The smaller peptide produced a greater response ($P < 0.05$) at each concentration examined. Values plotted are means \pm SEMs.

using aseptic technique under general anaesthesia induced and maintained by intravenous injection of sodium thiopental. Reproductive organs were exteriorized through a midventral abdominal incision. Ewes were treated post-operatively with a broad spectrum antibiotic.

Leucocyte migration assay

Leucocytes were isolated from the buffy coat of a plasma sample of an ovariectomized ewe, washed in Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY), and resuspended at 1×10^8 cells ml^{-1} . Migration of leucocytes toward conditioned media was assessed using a linear under-agarose assay described in detail by Murdoch and McCormick (1989). Briefly, a solution of HBSS containing 0.5% agarose and 0.5% BSA was pipetted onto microscope slides (55°C) and allowed to solidify at 4°C . Three circular wells 4 mm apart were cut in parallel from the agarose gel. Five microlitres of leucocytes was added into central wells and unconditioned media or experimental sample pipetted into flanking wells. Slides were incubated for 2 h in a

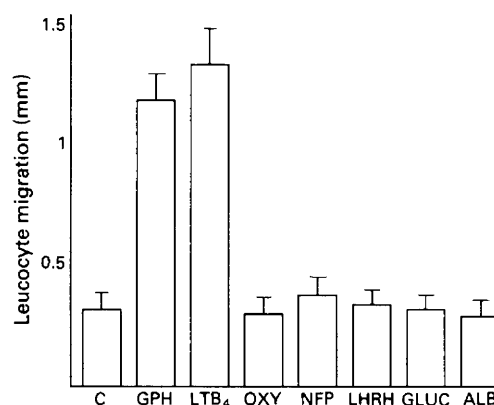


Fig. 3. Leucotactic effects of non-collagenous compounds versus GPH x 3 on migration of leucocytes under agarose. GPH x 3 and the known leucocyte chemoattractant, LTB₄ were effective ($P < 0.05$) in stimulating a response above control (C). OXY: oxytocin; NFP: N-formyl peptide; GLUC: glucagon; ALB: albumin.

humidified atmosphere at 37°C . Cells responding to a chemotactic gradient migrate toward that signal along the substratum between the slide and agarose. Leucocytes were affixed to the slide by immersion in 10% buffered formalin and the agarose gel was removed by inverting the slide. Cells were stained in Wright-Giemsa solution. Distances of migration of the leading front of the population of leucocytes toward wells containing conditioned media were determined with the aid of a micro-computer image-analysis system. Each sample was assayed in triplicate and these values averaged.

Amino acid analysis of leucocyte chemoattractant of follicular origin

The periovulatory follicle was dissected from surrounding ovarian tissues after unilateral ovariectomies carried out 24 ($n = 6$) and 36 ($n = 6$) h after injection of LHRH. A portion of follicular wall (about 4 mm^2) was placed in HBSS and incubated for 1 h at 37°C . Volume of incubation media was adjusted to weight of tissue ($4 \mu\text{l mg}^{-1}$). Conditioned media were pooled and subjected to ultrafiltration (5000 g for 2 h) through a Centricon 3000 molecular weight cut-off membrane (Amicon,

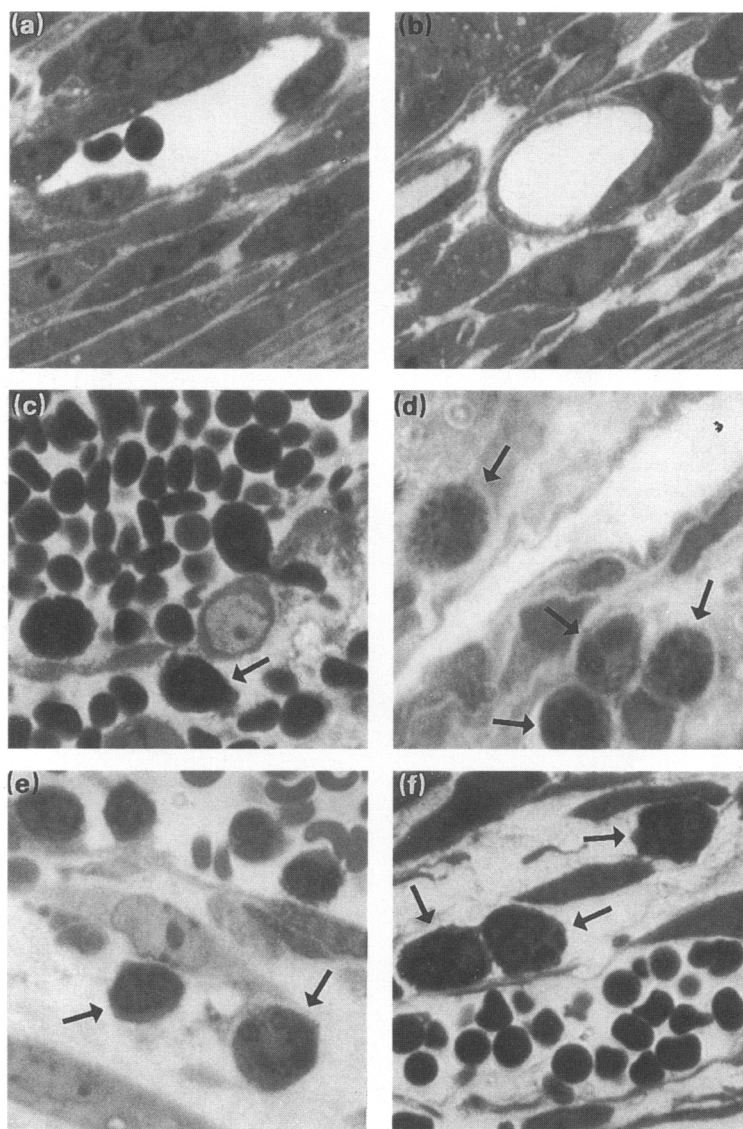


Fig. 4. Representative photomicrographs of histological sections of (a, b) control, (c, d) GPH $\times 3$ and (e, f) enzyme-treated follicles ($\times 1000$). A leucocyte caught in the process of migrating into the thecal interstitium is shown in (c). Extravascular white blood cells are denoted by arrows.

Danvers, MA). Filtrate was lyophilized and reconstituted in 0.1 ml distilled water.

The foregoing preparation was fractionated by binary gradient high performance liquid chromatography (HPLC) (McCormick *et al.*, 1988). A C-18 reverse phase, large pore size column was used. The primary solvent was 0.1% trifluoroacetic acid in water. Secondary solvent consisted of acetonitrile (AcN) containing 0.07% trifluoroacetic acid. Twenty microlitre aliquots of sample were applied to the column at 0% AcN at a flow rate of 1 ml min^{-1} at ambient temperature. The sample was eluted with a gradient of 0–50% AcN in 60 min. One microlitre eluate fractions were collected, dried by vacuum evaporation, reconstituted in 0.1 ml HBSS, and assayed for chemoattractant.

The fraction that exhibited bioactivity (retention time = 15 min) and bovine tendon type I collagen was processed for

analysis of both the imino and primary amino acids (Cunico *et al.*, 1986). Lyophilized samples were hydrolysed in acid (20 volumes w/v 6 mol HCl l^{-1} at 110°C for 24 h) and dried under vacuum. Hydrolysates were diluted once with distilled water and subsequently with $0.1 \text{ mol NaHCO}_3 \text{ l}^{-1}$ such that $5 \mu\text{l}$ would contain about 250 pmol of amino acid after derivatization and extraction. Samples were derivatized with 0.2 ml of $4 \text{ mmol 9-fluorenylmethylchloroformate l}^{-1}$ in dry acetone. Excess reagent was removed after a 10 min reaction by two extractions with equal volumes (0.4 ml) of pentane. After mixing and phase separation, pentane layers were discarded and $5 \mu\text{l}$ of the aqueous phase immediately injected onto the C-18 column. Separations were carried out using a ternary gradient system. The mobile phase consisted of $0.02 \text{ mol sodium acetate l}^{-1}$ (pH 2.85; Solvent A), Solvent A (pH 4.5) containing 20% methanol (Solvent B) and AcN (Solvent C). Amino acids

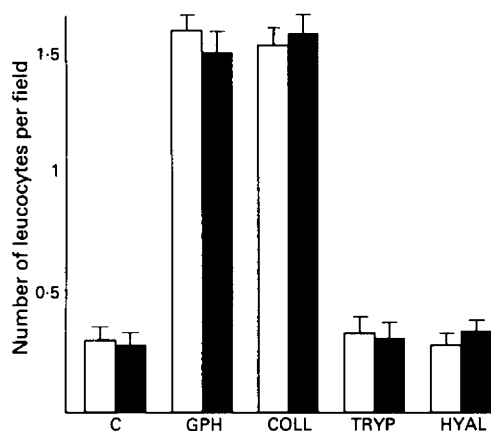


Fig. 5. Numbers of leucocytes (□: granulocytes; ■: mononuclear cells) within extravascular spaces of the theca interna 4 h after intrafollicular administration of injection vehicle control (C), GPH \times 3 or bacterial collagenase (COLL). Effects of GPH \times 3 and enzyme versus control were highly significant ($P < 0.01$). Trypsin (TRYP) and hyaluronidase (HYAL) were ineffective in causing leucocyte migrations.

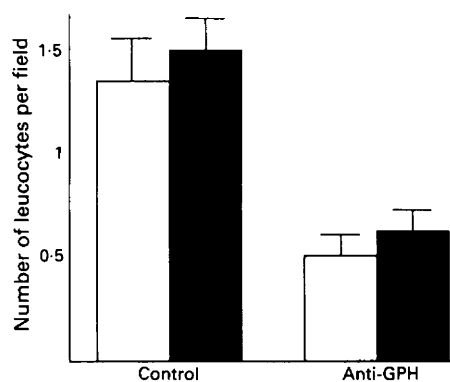


Fig. 6. Effect of preovulatory injection of intrafollicular anti-GPH \times 3 antibodies on thecal leucocytes (□: granulocytes; ■: mononuclear cells) of postovulatory follicles. Follicles were injected at 18 and collected at 36 h following administration of LHRH, respectively. Infiltration by both classes of leucocyte was inhibited ($P < 0.01$) by passive immunization.

were detected (sensitivity < 1 pmol) using a Hitachi F-1000 fluorescence spectrophotometer and peak areas were integrated using a Hitachi D-2000 recording integrator. Peak identities of unknown sample were confirmed by comparison of elution times with amino acid standards. Individual residues from averaged duplicate determinations are reported as a percentage of total amino acid hydrolysate.

Leucotactic activity of synthetic collagen-like peptides in vitro

Peptides consisting of a repeating sequence of glycine-proline-hydroxyproline (GPH \times 3, GPH \times 9) typical of endogenous peptide were custom synthesized (Multiple Peptide Systems, San Diego, CA; $\geq 80\%$ purity). Different concentrations of peptide (1, 10 and 100 nmol l^{-1} ; $n = 8$) were diluted in HBSS and tested for leucocyte chemotactic activity. Activity of GPH \times 3 at 100 nmol l^{-1} was compared with equimolar concentrations

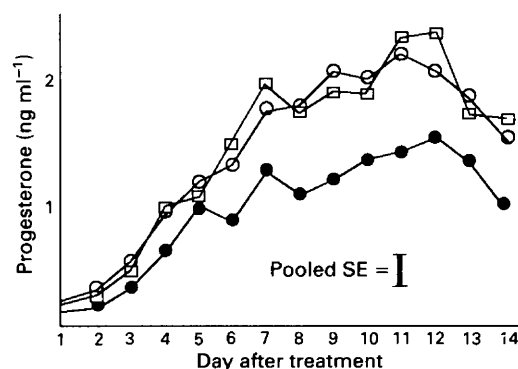


Fig. 7. Profiles of serum progesterone concentrations in (○) controls and (●) those ewes pretreated with intrafollicular anti-GPH \times 3 alone or in (□) combination with leucocytes. Amplitude of luteal activity was chronically depressed ($P < 0.05$) by anti-GPH \times 3 without leucocytes. Analogous data (not shown) were obtained from an initial experiment which included only control and anti-GPH \times 3 groups.

of leukotriene B_4 (LTB $_4$), *N*-formyl-L-noreleucyl-leucyl-phenylalanyl-noreleucyl-tyrosyl-lysine (*N*-formyl peptide), oxytocin, LHRH, glucagon and BSA ($n = 6$).

Effect of intrafollicular injection of collagen-like peptide or collagenase on thecal leucocyte extravasation

Ten micrograms of collagen-like peptide (GPH \times 3), bacterial collagenase (type 1A *Clostridium histolyticum*), trypsin (type I from bovine pancreas), hyaluronidase (type II from sheep testis) or vehicle (20 μ l of a solution of phosphate-buffered saline; PBS) were injected into the antrum of the dominant ovarian follicle of ewes (six per group) 36 h after administration of luteolysin (animals were not given LHRH at that time). Injections were made using a Hamilton syringe fitted with a 27-gauge hypodermic needle. The tip of the needle was directed into the follicular antrum through surrounding ovarian stroma. A block of tissue containing the preovulatory follicle was isolated following ovariectomy 4 h later. Tissues were fixed by immersion in chilled 10% buffered formalin, washed in PBS, dehydrated in a graded series of ethanol, cleared and infiltrated with paraffin. Embedded specimens were sectioned, rehydrated to water, and stained in haematoxylin and eosin. Granulocytes and mononuclear leucocytes were counted directly under the light microscope ($\times 1000$; about 0.06 mm 2). The lumen of a capillary or venule of the theca interna was orientated into the centre of the field of investigation. Ten sections and two areas within each section per follicle were chosen at random for morphometric analyses. Mean values for each animal were calculated.

Generation of antisera against GPH \times 3 and the effects of purified immunoglobulin on leucocyte chemoattraction in vivo and on ovulation and luteal function

Immunogen containing GPH \times 3 was prepared commercially (Multiple Peptide Systems). The free sulphhydryl group of cysteine-containing (*N*-terminal) peptide was coupled to key-hole limpet haemocyanin carrier protein via the bifunctional cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.

Antisera against conjugate was raised in a ram lamb. Primary and secondary immunizations given two weeks apart consisted of 250 µg conjugate emulsified in 6 ml of incomplete Freund's adjuvant. Subcutaneous injections, 1 ml, were made over six sites on the inside of the rear legs. Serum was collected by jugular venepuncture one week following the secondary boost. Antipeptide antibodies were purified from ammonium sulfate cuts of polyclonal serum by affinity chromatography using a kit supplied by Multiple Peptide Systems. The column matrix was made up of cross-linked agarose resin modified with *N*-hydroxyl succinimide ester. Sulfhydryl groups of the *N*-terminal cysteine residue were covalently attached to the agarose resin (1.5 ml) by reacting solubilized peptide (20 mg) in coupling solvent (0.5 ml) for 10 h at 25°C. The coupling reaction mixture (1.5 ml) was pipetted into the column, drained, and washed in column wash buffer. Any reactive ester groups remaining were neutralized by washing the column with 3 ml deactivating buffer. Saturated ammonium sulfate (0.5 ml) was mixed with crude serum (1 ml) and allowed to incubate on ice for 5 min. The solution was centrifuged at 10 000 *g* for 10 min at 4°C; the supernatant was decanted; and the pellet was resuspended in 2 ml of column wash buffer. The column was washed with low pH elution (2 ml) and washing (3 ml) buffers. Antibody solution was applied to the column and adsorbed immunoglobulin eluted by sequentially adding 1 ml aliquots of elution buffer. Fractions, 1 ml, were immediately neutralized with 0.3 ml neutralization buffer. Fractions yielding antibody (A_{280} determinations) were pooled and concentrated using a Centricon 30 000 molecular weight cut-off ultrafiltration device. Filtrate was reconstituted in PBS to a protein concentration of 2 µg µl⁻¹.

Antibody solution or PBS wash injected 18 h after LHRH (i.e. before expected leucocyte infiltration; Cavender and Murdoch, 1988) into the dominant follicle of ewes (five per group) using procedures described previously. Follicles were isolated 36 h after LHRH and subjected to histological analysis for white blood cell accumulation. This experiment was then repeated except that ovaries were examined by laparoscopy at 36 h, and to ensure that animals could only ovulate from the dominant follicle (i.e. form a single corpus luteum), any other antral follicles ≥ 3 mm in diameter were destroyed by electrocautery at the initial surgery. Blood samples were collected from the jugular vein each day for two weeks and analysed for serum progesterone by radioimmunoassay (Rahmanian and Murdoch, 1987). Finally, the latter study was replicated to include a group of ewes (*n* = 5) in which a suspension of leucocytes (2 × 10⁵) was co-injected into the dominant follicle with anti-GPH × 3 antibody.

Statistical analysis

Assignments to treatments were made at random. Significance of overall treatment effects was assessed using analysis of variance procedures. Specific means were contrasted with a protected least significant difference test or Student's *t* test.

Results

Amino acid analysis of the HPLC fraction exhibiting leucocyte chemoattractant activity revealed substantial amounts of

glycine and proline. Moderate levels of alanine, aspartate, glutamate and hydroxyproline were assayed. Other amino acids detected included arginine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine and valine. The fraction lacked cysteine, histidine and tyrosine (Fig. 1).

Leucocyte chemoattractant activity directed toward the synthetic collagen-like peptides was concentration dependent and related to size of peptide. Both peptides induced chemotaxis in a linear dose-related fashion. The shorter of the peptides (GPH × 3) was more active than the longer peptide (GPH × 9) at each concentration tested (Fig. 2). Activity elicited by the highest dose of GPH × 3 was similar to that of 100 nmol LTB₄ l⁻¹. Sheep leucocytes were not attracted toward non-collagenous proteins containing an identical (oxytocin), similar (*N*-formyl peptide, LHRH) or greater (glucagon, albumin) complement of amino acid residues than GPH × 3 (Fig. 3).

Local delivery of either GPH × 3 or bacterial collagenase into the preovulatory follicle stimulated the extravasation of white blood cells (Fig. 4). Leucocytes were confined mainly to the thecal layer and were not commonly observed within the membrana granulosa. There was evidence of some loss of integrity of the basement membrane in follicles that had been injected with collagenase. The magnitudes of response and respective degrees of accumulation of granulocytes and mononuclear cells due to type of treatment were similar. Intra-follicular injection of compounds unrelated to collagenase, namely trypsin or hyaluronidase, did not induce leucocyte migration into follicles (Fig. 5).

Injection of anti-GPH × 3 immunoglobulin into preovulatory follicles inhibited subsequent thecal leucocyte accumulation (Fig. 6). Nevertheless, on the basis of the presence of a follicular rupture point, ovulation had occurred in all antibody-treated and control ewes. Circulatory concentrations of progesterone were suppressed in ewes forming a corpus luteum from a follicle injected with antibodies. This effect was reversed by coincident intrafollicular injection of peripheral blood leucocytes (Fig. 7). Returns to oestrus following treatments were similar (15–17 days) among groups of ewes.

Discussion

Considerable information is available regarding the nature of the signalling processes involved in leucocyte migrations into tissue sites of generalized immune inflammatory reactions. A diverse number of compounds are known to attract white blood cells. Most notable among these are the complement component C5a, LTB₄ and the *N*-formylated peptides (Snyderman and Lane, 1989). Leukotriene B₄, but not *N*-formyl peptide was effective in attracting sheep white cells *in vitro*. Apparently leucocytes of some species lack *N*-formyl peptide receptors (Becker, 1983; Murdoch, 1987).

Very little is known concerning the makeup of leucocyte chemoattractants of ovarian origin. A neutrophil chemotactic protein was recovered from fluid of human ovarian follicles. It is heat labile, trypsin sensitive, and of high (about 100 kDa) molecular mass (Seow *et al.*, 1988). In contrast, as we have shown, ovine follicles mainly secreted a leucotactic protein of much lower molecular mass. This material was similar in amino acid composition to type I collagen isolated from bovine tendon

(present study) and human dermal collagen α_1 chain (Nimni and Harkness, 1988). The chemoattractant secreted by periovulatory ovine follicles also lacked tyrosine, histidine and cysteine, amino acids that occur sparingly or not at all in collagens.

In all probability, thecal collagen is degraded in a sequential manner. The initial rate-limiting step involves scission by a 'true' collagenase, that is, by a metalloproteinase that is active against native collagen fibrils at physiological pH. Once the helical conformation of collagen is thereby disrupted, the molecule can be progressively cleaved into small fragments by a variety of proteases (Harper, 1980). The possibility that products of follicular collagenolysis participate in leucocyte chemoattraction was corroborated by the experiment that established thecal infiltration of leucocytes following either intrafollicular administration of collagenase or synthetic collagen-like peptide. Indeed, synthetic collagen-like polypeptides which encompass the repeating triplets proline-proline-glycine or proline-hydroxyproline-glycine, and lacking helical structure, have been shown to possess chemoattractant properties toward human leucocytes *in vitro* (Laskin and Berg, 1986). Thus, it appears that the arrival of leucocytes into inflamed tissues, such as the ovulatory follicle (Espey, 1980), is mediated by the localized release of soluble chemotactic peptides from connective tissue. Whether follicular chemoattractant is definitely derived from thecal collagen is, however, still somewhat conjectural. For example, the complement sub-component C1q contains collagenous-like triplets of amino acids (Muller-Eberhard, 1988).

Activated leucocytes can produce a wide range of potent substances that could play a role yet to be defined in ovarian physiology. An intriguing recent report indicated that the ovulatory response to LH was enhanced when leucocytes were added to the perfusate of rat ovaries (Hellberg *et al.*, 1991). Neutrophils contain collagenases, elastases and chymotrypsins (Janoff, 1980). These proteolytic enzymes could conceivably contribute to the final stages of weakening of the follicular wall that precedes ovulation. Granulocytes also produce peroxidases (Gleich and Adolphson, 1986). Reactive oxygen species, generated in a burst upon cellular activation, can damage (and presumably weaken) inflamed tissue. Other proinflammatory agents secreted by leucocytes include prostaglandins, leukotrienes (Janniger and Racis, 1987), platelet-activating factor (Braquet *et al.*, 1987) and various cytokines (for example, interleukins, tumour necrosis factors and interferons) (Harrison and Campbell, 1988). Certainly metabolites of arachidonate have assumed a central role in the mechanism of ovulation (Goetz *et al.*, 1991). Our study indicates that leucocytes attracted into the follicle from the vascular system are not required for successful ovulation in sheep. Perhaps leucocytes can augment the ovulatory reaction in cases of multiple ovulation.

After follicular rupture and release of the maturing oocyte, the ovulated follicle is transformed into a corpus luteum. This event is characterized by cellular reorganization and neovascularization. Given that luteal dysfunction is a sequel to inhibition of follicular leucocyte migration, it seems plausible that growth and angiogenic factors (along with proteases) derived from leucocytes might aid in the appropriate remodelling of a follicle during the luteinization process (Hammond *et al.*, 1988; Koos, 1989).

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