0021-972X/94/7902-0633$03.00/0
Journal of Clinical Endocrinology and Metabolism
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Vol. 79, No. 2
Printed in U.S.A.

Interleukin-6 Biosynthesis in Human Preovulatory Follicles: Some of Its Potential Roles at Ovulation

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ABSTRACT

In the present work we explored cellular sites of interleukin-6 (IL-6) biosynthesis in human follicular aspirates from patients undergoing *in vitro* fertilization therapy and the effects of this cytokine on oocyte fertilization and granulosa cell (GC) steroidogenesis.

Biological IL-6 activity from 20–40 IU/mL was present in follicular fluids from 22 patients; it was also detected in 10 of 22 supernatants of cultured oocyte-cumulus complexes and in cumulus cell and GC cultures. Biological IL-6 activity in oocyte-cumulus complex cultures was not related to fertilization rates.

Total ribonucleic acid was isolated from follicular aspirates and GC-enriched preparations. After reverse transcriptase and polymerase chain reaction cycles using oligonucleotide primers corresponding to known cDNA sequences for IL-6, a 126-basepair band characterized the amplification product of IL-6 transcripts on gel electrophoresis. To localize IL-6 messenger ribonucleic acid, *in situ* hybridization analysis was performed using a [^{35}S]IL-6 riboprobe. The distribution of transcripts was more dense (15% *vs.* 3% stained cells) in GC-enriched preparations, which contained more than 95% GCs, than in original follicular preparations, which contained 20–40% viable GCs; it was not significantly modified by the presence of macrophage contaminants. The expression of IL-6 protein was assessed by positive immunohistological stainings. Biological IL-6 activity was higher, and *in situ* hybridization signals were more dense and more intense in 24-h GC cultures than in 72-h GC cultures, suggesting that IL-6 biosynthesis was transiently induced. Under experimental conditions of low IL-6 endogenous levels in cultures, adding recombinant human IL-6 from 10–200 IU/mL had no effect on progesterone production or aromatase activity in GC cultures free of macrophages, whereas in GC cultures including macrophage contaminants, stimulatory effects on basal and hCG-stimulated progesterone production and on basal and FSH-stimulated aromatase activities were observed.

The present study provides strong support for the view that IL-6 is produced by GCs in the preovulatory follicle at the time of ovulation. In addition, we showed that IL-6 might be an intraovarian regulatory factor concerned with steroidogenesis. (*J Clin Endocrinol Metab* **79:** 633–642, 1994)

IN RECENT years, evidence has accumulated to suggest that the immune system is an additional local regulator of ovarian function, involving reciprocity among soluble polypeptides of immunological origin, the cytokines, and the reproductive system. Numerous studies have shown that a variety of cytokines are capable of exerting profound effects on ovarian function and are the probable mediators of immune influence on reproductive processes, implicated as regulators of gonadal steroid secretion, corpus luteum function, embryo development, and implantation (1). Interleukin-6 (IL-6) is involved in numerous immunological, proliferative, and neoplasic processes (2), including follicular angiogenesis (3). More recently, evidence has emerged which demonstrates that IL-6 can directly influence ovarian function (4). IL-6 is synthesized by a variety of cell types that play important roles in both immunity and inflammation. IL-6 production is not constitutive, but is transiently induced by various stimuli, including the inflammatory process. Ovulation, initiated by the LH surge, completes an inflammatory-like process (5), triggering massive ovarian infiltration by representatives of the white blood cell series in the preovulatory follicle. Examination of follicular fluid (FF) from *in vitro* fertilization (IVF) patients revealed the presence of lymphocyte, T-cell, and monocyte subpopulations (6, 7) and significant amounts of IL-6-like activity (8, 9). Various cell types, including granulosa cells (GCs), can be sources of IL-6 (10, 11). In the present study we explored cellular sites of IL-6 biosynthesis in human follicular aspirates from patients undergoing IVF therapy. Particularly, we examined whether GCs could produce IL-6 in the preovulatory follicle. The microenvironment of human follicles is vital for normal oocyte development. Cytokines, including IL-6, have been suggested to influence oocyte fecundity (9). Here, we explored the presence of IL-6 in oocyte-cumulus complex (OCC) cultures in relation with oocyte fertilization rates. Among the broad spectrum of effects that IL-6 might mediate in relation to ovulation-associated phenomena, we studied its role in GC steroidogenesis by autocrine and paracrine effects via macrophages.

Received September 27, 1993. Revision received April 1, 1994. Accepted April 14, 1994.

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Materials and Methods

Stimulation protocol and specimen collection

The material for this study was obtained from patients (aged 20–42 yr) undergoing IVF-embryo transfer therapy. The study required no modification of routine human IVF protocols. Briefly, the patients were treated with human menopausal gonadotropin under medical hypophy-

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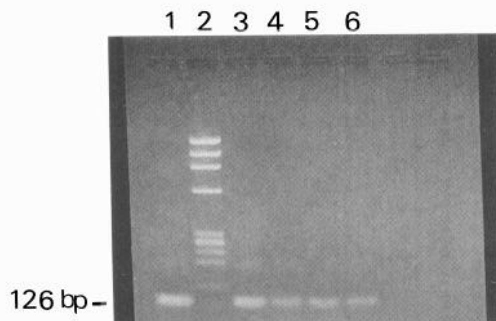

FIG. 1. PCR products obtained from total RNA isolated from human follicular aspirates of four patients undergoing IVF (lanes 3–6) and by cultured GC from pooled aspirates (lane 1); lane 2 is a DNA ladder. After RT reaction and PCR cycles using oligonucleotide primers corresponding to known cDNA sequences for IL-6, the samples were subjected to agarose gel electrophoresis, and the gels were stained with ethidium bromide. Multimers of a 126-basepair (bp) fragment of DNA characterized the amplification product of IL-6 transcripts.

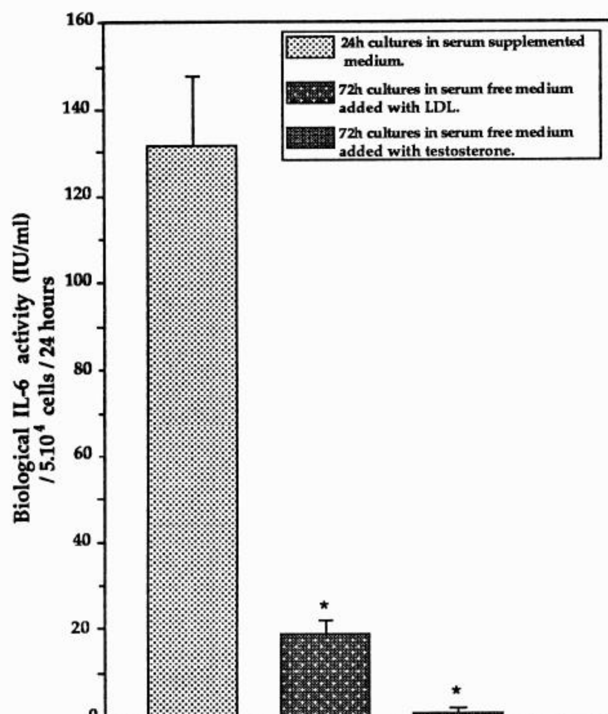

FIG. 2. IL-6 levels measured by B9 cell proliferation assay in cultures of human GC treated to remove blood cell contaminants. Histograms represent successive IL-6 levels in 24-h cultures in serum-containing medium, IL-6 levels in 72-h cultures in serum-free medium with 50 μg/mL LDL, and IL-6 levels in 72-h cultures in serum-free medium with 10^{-3} mol/L testosterone. Biological IL-6 activity was measured for 24 h and expressed as international units per mL/5×10^4 cells. The assays were repeated on four series of cultures from different follicular aspirates. Data are the mean ± SE. ★, $P < 0.01$, by Scheffe's test after one-way analysis of variance, 24-h serum-containing cultures *vs.* 72-h serum-free cultures and 72-h serum-free cultures with LDL *vs.* 72-h serum-free cultures with testosterone.

sectomy induced by GnRH agonists, Decapeptyl (Ipsen Biotech, Paris, France) or Buserelin acetate (Suprefact, Hoechst Laboratories, Paris, France), for induction of multiple follicles. Granulosa cells and OCC were aspirated from individual follicles under ultrasonographic control approximately 36 h after the injection of 10,000 IU hCG (Profasi, Serono), a time considered day 0 in the subsequent experiments. FF samples were stored at −20 C before assaying. Blood samples were recovered on the day of oocyte retrieval. Part of each sample was spun to separate serum, which was kept frozen until assayed; from the other part, human peripheral monocytes were isolated by allowing the mononuclear phagocytes to adhere to dishes.

OCC recovery and culture

OCC were cultured in 0.5 mL Inra-Menezo-B_2 medium (Biomerieux Laboratories, Marcy l'Etoile, France) for 20–24 h in the presence of 20,000 washed motile sperm. After 1 day in culture, residual cumulus was detached from oocyte using thin needles, then media were pooled for each patient and centrifuged. The supernatants (day 1 medium) were collected and frozen at −20 C until assayed, and cumulus cells collected from the pellet were cultured. After the second day of oocyte culture in fresh B_2 medium, the collected medium was centrifuged and frozen until assayed (day 2 medium). Control medium was obtained from sperm incubated in B2 medium for 24 h.

Cumulus cells were inoculated in 24-well tissue plates at 5×10^4 cells/well in 1 mL Ham's F-12-Dulbecco's Minimum Essential Medium (1:1; Gibco-BRL, Eraguy, France) buffered with 15 mmol/L HEPES, also including 0.365 g/L L-glutamine, 50 μg streptomycin, and 50 IU/mL penicillin, supplemented with 5% heat-inactivated fetal calf serum (Gibco-BRL), then incubated in an humidified atmosphere of 5% CO_2 at 37 C. Culture medium was daily removed.

GC-enriched preparations from follicular aspirates

After removal of OCC, follicular aspirates were pooled and centrifuged at $200 \times g$ for 5 min and resuspended in phosphate buffer solution (PBS), then layered on a Percoll cushion (1:1, PBS-Percoll; Pharmacia, St. Quentin-Yvelines, France) and centrifuged at $1000 \times g$ for 20 min to pellet red blood cells. Cells aspirated from the interface contained 6–7% monocytes/macrophages, 10–12% lymphocytes (10), and 40–60% dead cells, as shown by the dye exclusion test using trypan blue (Sigma Chemical Co., St. Quentin-Fallauier, France). Viable granulosa cells represented only 20–40% of the cell population in these original preparations. Blood cell contaminants were eliminated by successive steps, including salt treatment (10) to remove lymphocytes and immunobead treatment to eliminate macrophages. To remove macrophages, cells were incubated for 10 min at room temperature with immunomagnetic beads (Immunotech, Marseille, France) coupled to the monoclonal antibody CD68 (Ki M7, Behring, Dako-Trappes, France). Then, the incubate was placed into a magnetic test tube rack for 10 min at room temperature. After careful removal of the supernatant, the process was repeated twice under identical experimental conditions to ensure complete removal of target cells. Then, cells from the resultant preparations were plated in Ham's F-12-Dulbecco's Minimum Essential Medium (1:1; Gibco-BRL) buffered with 15 mmol/L HEPES, also including 0.365 g/L L-glutamine, 50 μg streptomycin, and 50 IU/mL penicillin, supplemented with 5% fetal calf serum (FCS; Gibco-BRL). After a 24-h culture period, non-adherent cells were eliminated by successive washings; adherent cells, mostly GC, characterized by their typical morphology, exhibiting accumulations of lipid droplets and granules in their cytoplasm, were detached from plastic support using a 1-fold concentrated trypsin-EDTA solution (Gibco-BRL), then washed extensively with 1-fold concentrated PBS. Their viability was more than 95%, and the absence of monocyte/macrophages, which could adhere to culture support and contaminate GC cultures, was controlled by immunostaining. In addition, neither fibroblast nor endothelial cells, which are potent sources of IL-6, were observed in our cultures. Thus, we could obtain, after a 24-h culture period, enriched GC preparations that contained more than 95% granulosa cells, as assessed by their 3β-hydroxydehydrogenase activity which was determined by the development of a black blue precipitate within the cells, using a procedure previously described (11). Some additional GC-enriched preparations were completed after a 72-h culture period in serum-containing medium and serum-free medium in either the presence or absence of hCG. To obtain GC-enriched preparations including macrophage contaminants, we omitted immunobead treatment, and the presence of macrophages was determined by immunostaining.

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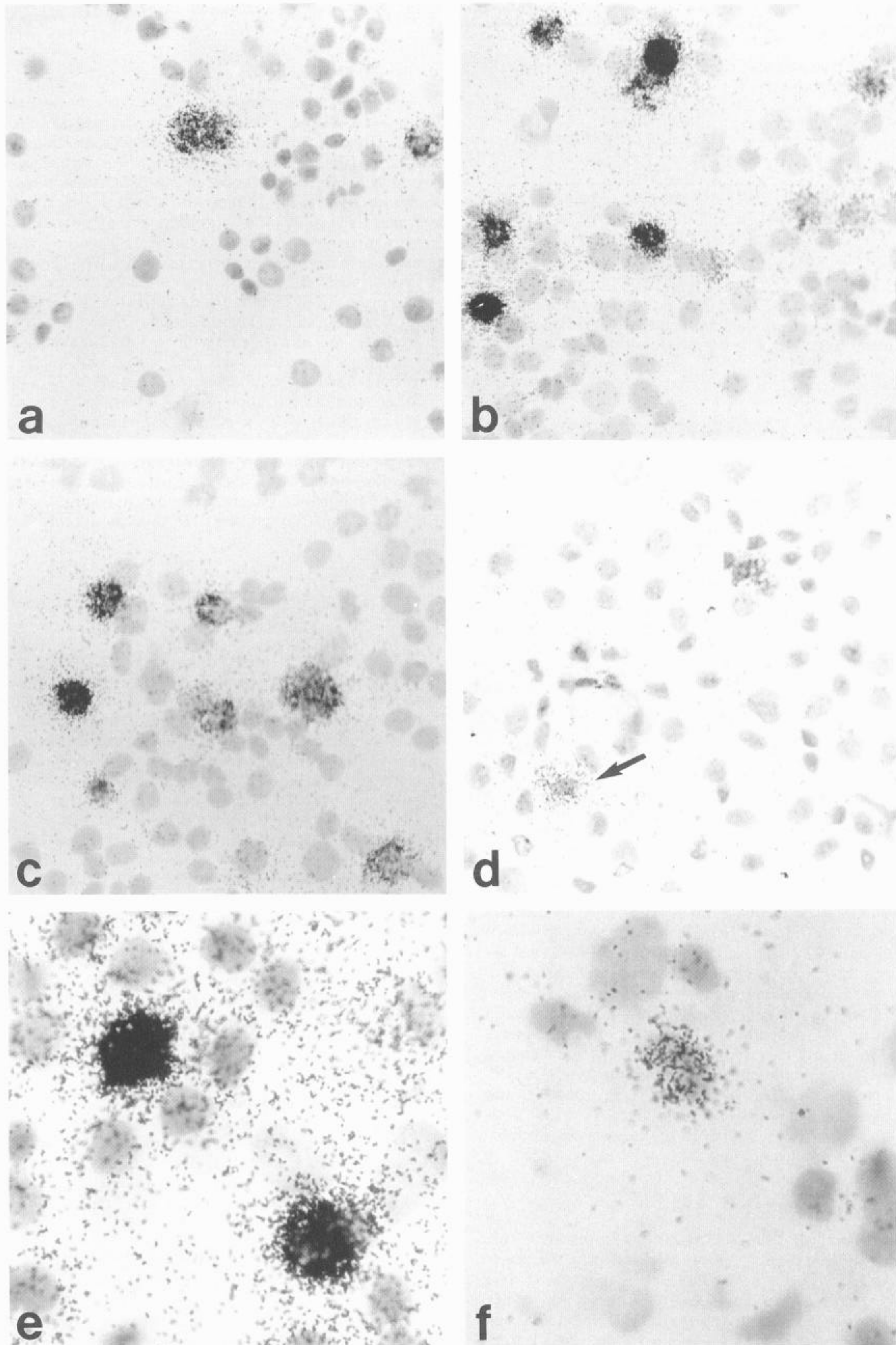

FIG. 3. *In situ* hybridization of a ^{35}S-labeled human IL-6 riboprobe. a, Original preparation containing 20–40% GC. Magnification, ×300. b, Enriched GC preparations not treated to remove macrophages; these preparations included more than 95% GC and 3–4% macrophage contaminants. Magnification, ×300. c, Preparations enriched in GC (>95%) and cleared of macrophages. Magnification, ×300. d, Enriched GC preparations after a 72-h culture period (magnification, ×300). e, Density of silver grains on reactive cells from an enriched GC preparation after a 24-h culture period. Magnification, ×600 (oil immersion). f, Density of silver grains on reactive cells from enriched GC preparations after a 72-h culture period. Magnification, ×600 (oil immersion). Reactive cells contain clusters of silver grains, showing the presence of IL-6 mRNA in these cells. Cells were counterstained with hematoxylin, which stained nuclei.

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TABLE 1. Biological IL-6 activity in FF and culture medium of OCC from 22 patients undergoing IVF

No. of patients	IL-6 (IU/mL)		Progesterone (ng/mL)	Fertilization rates
	FF	OCC	OCC	
12	35 ± 12	ND	289 ± 75	0.836 ± 0.06
10	29 ± 7	21 ± 5	394 ± 69	0.704 ± 0.03
P value (by unpaired Student's *t* test)	NS		NS	NS

The presence and absence of IL-6 in OCC cultures were related to progesterone levels in OCC culture medium and to fertilization rates.
Values are the mean ± SE. NS, $P > 0.05$; ND, not detectable.

GC cultures

GC cleared of blood cell contaminants and GC still including macrophage contaminants were inoculated in 24-well plates at 5×10^4 cells/well in medium containing 5% FCS. After 24 h to allow attachment and recovery from the effects of trypsin, medium was removed and replaced with serum-free medium for 72 h. Progesterone (P) production was measured in medium containing 50 μg/mL low density protein (LDL) isolated from human serum; after a 48-h culture period, hCG (Sigma; SA, 15×10^3 IU/mg) or dibutyryl cAMP [$(Bu)_2$cAMP; Sigma] was added to cultures for 24 h. Aromatase activity was assayed after a 72-h culture period in serum-free medium containing 10^{-3} mol/L testosterone (Sigma) in the absence and presence of FSH (Sigma; SA, 7000 IU/mg) or $(Bu)_2$cAMP. To determine the effects of IL-6 in cultures, human recombinant IL-6 (rhIL-6; Genzyme; SA, 4×10^7 U/mL, as determined by the B9 cell proliferation assay) was added to culture medium.

Hormone and cytokine assays

P and estradiol (E_2) were assayed by RIA, using COATRIA kits (Biomérieux Laboratories, Marcy l'Etoile, France). The assay sensitivities were 0.65 ng/mL and 12 pg/mL for P and E_2, respectively; intra- and interassay coefficients of variation were 6% and 14%, respectively. Aromatase activity was assessed by measuring the release of ^3H_2O from 1β-[^{3}H]androstenedione (27.4 Ci/mmol/L; Du Pont de Nemours, Dreieich, Germany). GC were incubated at 37 C for 5 h in medium containing an excess of 1β-[^{3}H]androstenedione equivalent to a final concentration of 0.5 μmol/L. Nonspecific production of ^3H_2O was assessed by incubating 1β-[^{3}H]androstenedione in the absence of GC. Aromatase activity was expressed in picomoles per mL androstenedione converted per 5 h. IL-6 was assayed using the murine hybridoma cell line B9, selected for its IL-6 dependence (12). All of the supernatants tested were titrated in serial 2-fold dilutions and related to a standard recombinant human IL-6 preparation. Cells were pulsed for 6 h with [^{3}H]thymidine using 7.4 kBq [^{3}H]thymidine (74 GBq/mmol/L), and incorporation was assessed. Assay sensitivity was about 5–10 IU/mL; as a consequence, IL-6 activity lower than 5–10 IU/mL was undetectable under the present experimental conditions.

Immunohistological techniques

Macrophages were localized using the monoclonal antibody CD68, diluted 1:50, and cellular IL-6 production was localized using the monoclonal antibody anti-IL-6 (13), diluted 1:100. Before staining, cells were cytospun, air dried, and fixed in 4% formalin in PBS for 30 min at 4 C. The antibody complex was visualized with the indirect immunoperoxidase method using Vectastain ABC kits (Vector Laboratories, Burlingame, CA). Cells were counterstained with hematoxylin, mounted with glycerin PBS, and observed under the light microscope. The presence of an easily detectable red to brownish-red stain indicated positive binding by the primary antibody. The negative controls included both omitting the primary clonal antibody and using preimmune sera instead of primary antibody.

In situ hybridization

This was performed essentially as previously described (14). As a source of IL-6-specific sequences, we used a complementary DNA (cDNA) clone of human IL-6. ^{35}S-Labeled complementary ribonucleic acid (cRNA) in an antisense orientation was synthesized *in vitro* and used as a hybridization probe. In brief, GC were cytospun, air dried, fixed with acetone for 10 min, and stored at −80 C until used. After acetylation, cell spots were hybridized with a ^{35}S-labeled antisense RNA probe that was complimentary to nucleotides of the coding sequence of human IL-6 in a solution containing 50% (vol/vol) deionized formamide, 0.3 mol/L NaCl, 10 mmol/L Tris (pH 8.2), 1 mmol/L EDTA, 0.05% yeast transfer RNA, 10 mmol/L dithiothreitol, 1 × Denhardt's solution, and 10% dextran sulfate. Hybridization solution (30 μL) was placed over each cellular spot and covered with a 22 × 22-mm acid-washed siliconized coverslip. Coverslips were then sealed with rubber cement. Cells were hybridized by incubation overnight at 55 C in an humidified chamber. After hybridization, the slides were treated with ribonuclease-A (520 μg/mL) at 37 C for 30 min, washed in buffers with decreasing salt concentrations, dehydrated through ethanol gradients, and processed for liquid emulsion autoradiography (Kodak NTB-2, Eastman Kodak, Rochester, NY). Slides were exposed for 4 weeks at 4 C, developed, and stained with hematoxylin before being examined and photographed. Sense RNA probes were used as a control for nonspecific binding.

Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of IL-6 messenger RNA (mRNA)

IL-6 mRNA was transcribed into cDNA using RT, followed by amplification of the cDNA using PCR. Pellets of about 2×10^6 cells were lysed in RNA extraction buffer using the RNAzol technique (Bioprobe Systems, Montreuil, France). The mixture was incubated for 5 min at 4 C, then extracted with chloroform (0.1%, vol/vol), and RNA was precipitated with isopropanol (vol/vol). The pellet was resuspended in 200 μL water and frozen at −80 C. Aliquots of RNA were then treated at 37 C for 30 min with 10 U DNAse (Boehringer, Bagnolet, France) in the presence of 10 U human placental ribonuclease inhibitor (Boehringer), then extracted with phenol-chloroform and precipitated in ethanol. RNA was transcribed into cDNA by incubation in 10 μL 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.5 mmol/L deoxy-ATP, 0.5 mmol/L deoxy-CTP, 0.5 mmol/L deoxy-GTP, 0.5 mmol/L deoxy-TTP, 10 pmol/L antisense IL-6 primer (5′-CTGGAGGTACTCCCCT-AGGGGGTAATAC-3′, from bases 476–455), 20 U human placental ribonuclease inhibitor (RNAsin), and 200 U Moloney murine leukemia virus RT (BRL, Cergy-Pontoise, France). RT reactions were performed at 42 C for 1 h, then samples were heated to 70 C for 5 min and cooled to 4 C. For PCR amplification of the cDNA products, 10 μL reaction product were mixed with 20 pmol/L antisense primer and 30 pmol/L sense primer (5′-TCAAATGAGGAGACTTGCCTG-3′, from bases 350–370), 1.25 mmol/L deoxynucleotides phosphates, 8 μL 10 × PCR buffer, and 2.5 U *Taq* DNA polymerase (BioTaq, Bioprobe Systems, Montreuil, France), and the volume was adjusted to 50 μL. The samples were then subjected to PCR cycles by setting the denaturation, annealing, and extension temperatures to 94 C (for 1 min), 55 C (for 1 min), and 72 C (for 1 min), respectively. After 30 cycles, the samples were run on agarose gels containing ethidium bromide (Sigma), and the PCR products were photographed.

Statistical analysis

Values for P, aromatase, and IL-6 activities in cultures were measured per well in quadruplicate and expressed for 5×10^4 viable cells initially inoculated. Experiments were repeated four times on different original suspensions, and variations were expressed as the mean ± SE. Statistical significance was determined on a Macintosh SE/30 computer (Apple Computers), using one-way analysis of variance, followed by Scheffe's F test. Student's *t* test for unpaired data was used to determine statistical differences between two groups of patients.

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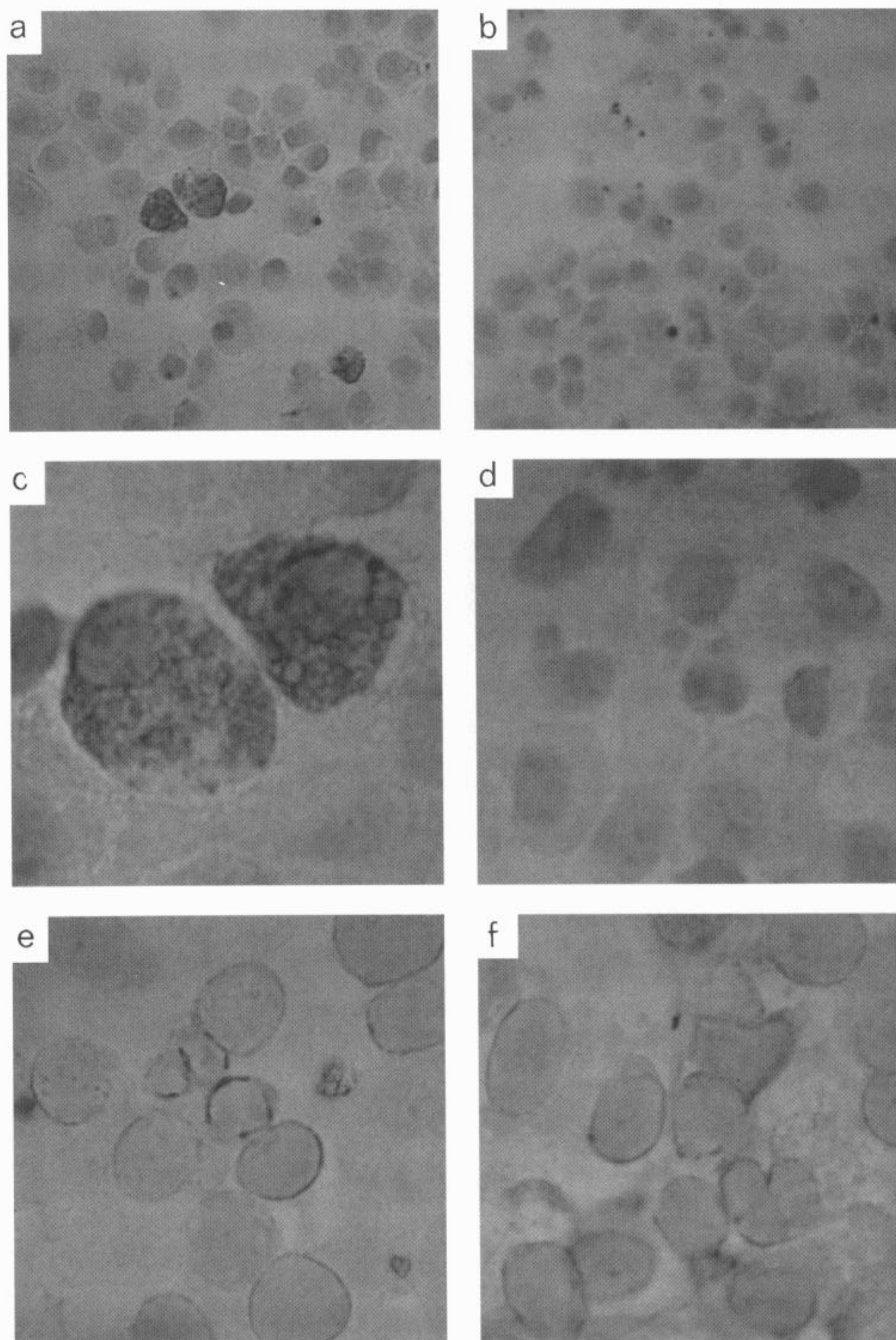

FIG. 4. Immunocytochemical identification of macrophages and IL-6 sites of production. a, Cells colored with the monoclonal CD68 antibody from GC-enriched preparations that were not treated to remove macrophages. Magnification, ×300. b, Cells from preparations treated to remove macrophages; no cell was colored after immunostaining with the CD68 antibody. Magnification, ×300. c, Immunostaining of macrophages observed at a magnification of ×1200 (oil immersion). d, Negative control in the absence of primary antibody, at a magnification of ×900 (oil immersion). e, Immunocytochemical localization of IL-6 on cell spots from GC-enriched preparations including macrophage contaminants. Magnification, ×900 (oil immersion). f, Immunocytochemical localization of IL-6 on cell spots from GC-enriched preparations cleared of macrophages. Magnification, ×900 (oil immersion). Reddish brown immunostain represents cell sites of specific immunoreactivity. Cells were counterstained with hematoxylin, which produced the blue stain of the nuclei.

Results

Biological IL-6 activity ranging from 20–50 IU/mL was detected in FF sample from 22 patients. For 6 of the 22 patients, IL-6 concentrations were measured in parallel in FF and serum samples collected on the day of oocyte retrieval. Biological IL-6 activity was nearly undetectable in serum samples.

Total RNA was extracted from follicular aspirates from four patients and from GC-enriched preparations, then treated with RT-PCR techniques. A 126-basepair band characterizing amplification products of IL-6 transcripts was shown on the electrophoresis gels for every sample examined (Fig. 1). No band was detected for peripheral monocytes. Negative controls were performed with samples not treated with RT.

Biological IL-6 activity in GC cultures

After determining that medium supplemented in FCS did not contain any trace of IL-6, biological IL-6 activity was

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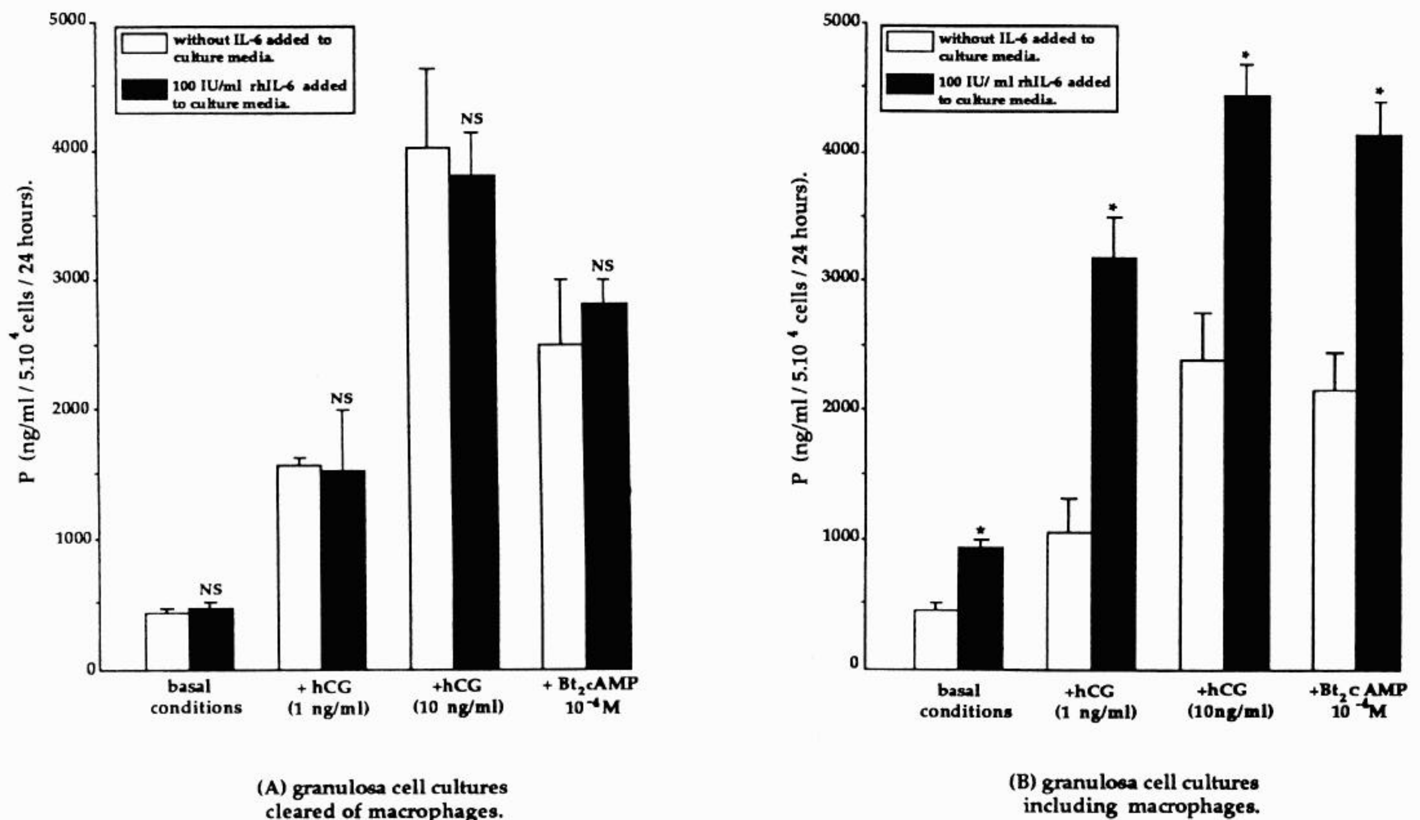

FIG. 5. Effects of IL-6 on P production in human GC cultures free of macrophages (A) and in GC cultures including macrophage contaminants (B). Human rhIL-6 at a dose of 100 IU/mL was added for 24 h to serum-free medium supplemented with LDL under basal conditions and in the presence of 1 or 10 ng/mL hCG or 10^{-4} mol/L $(Bu)_2cAMP$. Controls were completed in cultures without rhIL-6. P was assayed in 24-h culture medium samples. The experiment was repeated in four series of cultures obtained from different aspirates. Data are the mean ± SE. ★, $P < 0.01$; NS, $P > 0.05$ (by Scheffe's test after one-way analysis of variance, *vs.* cultures without rhIL-6). In cultures cleared of macrophages (A), basal P production and the percent increment in P production induced by hCG or $(Bu)_2cAMP$ were not modified by adding rhIL-6 to the culture medium, whereas in cultures including macrophages (B), P levels was significantly increased in cultures supplemented with rhIL-6.

assayed in cultures. No significant differences were detected between GC cultures cleared of macrophages and GC cultures not treated with immunobeads and still containing macrophages. At the end of a 72-h culture period, IL-6 levels significantly decreased *vs.* levels detected in 24-h cultures. In addition, IL-6 levels were lower in cultures with testosterone than in cultures with LDL (Fig. 2). The levels of E_2 and P were measured in cultures with LDL and cultures with testosterone; they were, respectively, 5×10^3 pmol/L and 4.6×10^4 nmol/L in cultures with LDL and 2.5×10^5 pmol/L and 8.4×10^2 nmol/L in cultures with testosterone. The addition of hCG or FSH did not significantly modify IL-6 activity in the cultures.

Determination of cellular sites of IL-6 biosynthesis

In situ hybridization techniques were performed on cell spots from original preparations including 20–40% GC (Fig. 3a) and on cell spots from GC-enriched preparations containing more than 95% GC (Fig. 3, b and c). Cellular localization of IL-6 transcripts clearly identified sites of IL-6 synthesis. Their relative number was 15% in GC-enriched preparations *vs.* 3% in original preparations, showing that the higher the number of granulosa cells, the higher the number of cellular sites of IL-6 biosynthesis. The distributions of IL-6 transcripts were similar in GC-enriched preparations with 3–4% macrophage contaminants (Fig. 3b) and preparations cleared of macrophages (Fig. 3c). Macrophages specifically colored with CD68 antibody in preparations not treated with immunomagnetic beads (Fig. 4, a and c), whereas no staining was detected in preparations treated with immunomagnetic beads (Fig. 4b). Immunocytochemical staining with IL-6 antibody was positive on GC-enriched preparations still containing macrophages (Fig. 4e) and those cleared of macrophages (Fig. 4f). No staining was detected in the absence of primary antibody (Fig. 4d).

After a 72-h culture period, the relative number of cells labeled by *in situ* hybridization techniques dropped to 2–3% (Fig. 3d) *vs.* 15% in 24-h cultures (Fig. 3c), and the density

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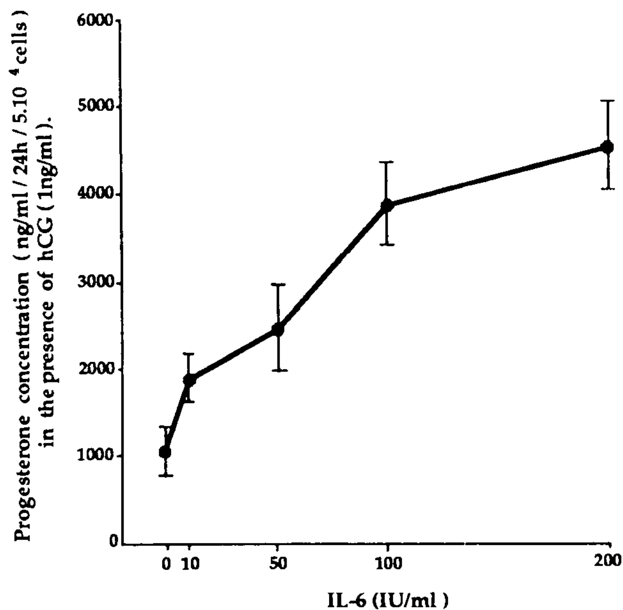

FIG. 6. Effects of increasing rhIL-6 doses (0, 10, 50, 100, and 200 IU/mL) added to culture medium for 24 h on hCG-stimulated P production in GC cultures including macrophages. Experiments were completed in serum-free medium supplemented with 50 μg/mL LDL in the presence of 1 ng/mL hCG; they were repeated in two series of cultures from different follicular aspirates. Data are the mean ± SE.

of grains per cell was less intense (Fig. 3f) than that in 24-h cultures (Fig. 3e). The presence or absence of serum in culture medium as well as adding or not adding hCG for 72 h did not modify levels of IL-6 transcripts. No specific staining was detected with the sense strand (control) probe.

IL-6 and oocyte fertilization rates

Biological IL-6-like activity was explored in OCC cultures (day 1 medium) and denuded oocyte-embryo supernatants (day 2 medium) from 22 patients. OCC supernatants from 10 patients displayed detectable IL-6 activity, whereas that of 12 patients did not show any measurable IL-6 activity, although P levels in OCC culture medium and fertilization rates were similar ($P > 0.05$, by Student's t test; Table 1). Control sperm medium and denuded oocyte-embryo supernatants (day 2 medium) showed no IL-6 activity. Slight IL-6 activity from 10–30 IU/mL was detected in 24-h cultures of cumulus cells from 5 patients. These levels were significantly lower than those detected in GC cultures under similar experimental conditions (10–30 *vs.* 110–150 IU/mL).

IL-6 and GC steroidogenesis

The effects of IL-6 on GC steroidogenesis were tested in 72-h serum-free cultures with low endogenous IL-6 activity. The effects of IL-6 were determined in GC cultures cleared of macrophages and in GC cultures including macrophage contaminants from four series of pooled follicular aspirates. In both types of cultures, P levels rose in the presence of 10^{-4} mol/L $(Bu)_2cAMP$ or 10 ng/mL hCG added for 24 h, and this increase was dose dependent for hCG doses from 0–10 ng/mL. Adding 100 IU/mL rhIL-6 for 24 h to GC cultures cleared of macrophages had no effect on either basal P production or P production stimulated by hCG or $(Bu)_2cAMP$. However, adding 100 IU/mL IL-6 for 24 h to GC cultures including macrophages significantly increased P production in the absence and presence of hCG or $(Bu)_2cAMP$ ($P < 0.01$, by Scheffe's test; Fig. 5). This augmentation evoked a significant dose-dependent increase in stimulated P release when various concentrations of IL-6 (10, 50, 100, and 200 IU/mL) were added to a fixed concentration of 1 ng/mL hCG (Fig. 6).

Concurrent exposure to 100 IU/mL rhIL-6 in GC cultures cleared of macrophages had no effect on aromatase activities in either the absence or presence of 10 ng/mL FSH or 10^{-4} mol/L $(Bu)_2cAMP$ ($P > 0.05$, by Scheffe's test). However, adding 100 IU/mL IL-6 to GC cultures containing macrophages significantly increased aromatase activities under basal conditions and in the presence of 1 and 10 ng/mL FSH and 10^{-4} mol/L $(Bu)_2cAMP$ ($P < 0.01$, by Scheffe's test; Fig. 7).

Discussion

Evidence of IL-6 in preovulatory follicles was previously reported (8, 9). Here, IL-6-like activity ranging from 20–50 IU/mL was detected in 22 patient FF, whereas IL-6 activity was nearly undetectable in serum samples collected from 6 patients on the day of oocyte recovery. Because IL-6 is removed from the circulation with a half-life of about 3 min (15), IL-6 could be produced locally in the preovulatory follicle, whereas it was not detected in serum. Under physiological conditions, the low IL-6 levels detected in serum are at the limit of sensitivity of the biological assay we used here. Higher levels of circulating IL-6 are usually correlated with tissue damage and pathology, as recently demonstrated for women with ovarian hyperstimulation syndrome and patients with ovarian epithelial cancer (16, 17).

RNA extracted from follicular aspirates and GC-enriched preparations, reverse transcribed, then treated with PCR reactions containing oligonucleotides primers for IL-6, showed a characteristic 126-basepair band corresponding to amplification products of IL-6 transcripts. Cellular localization of IL-6 mRNA on cell spots by *in situ* hybridization allowed determination of the relative number of active sites of IL-6 synthesis. These sites were more numerous in enriched GC preparations from 24-h cultures (15% of stained cells) including more than 95% granulosa cells than on the original preparation from follicular aspirates (3% of stained cells), including 20–40% viable granulosa cells. Thus, the increase in the expression of IL-6 transcripts followed the enrichment of granulosa cells; 20–40% of viable GCs in original cell preparations *vs.* more than 95% in enriched GC preparations. Even if unidentified contaminant cells might contaminate enriched GC preparations, it was only in the range of 1–5% under our experimental conditions, and in any case they could be responsible for the labeling of 15% cells. On the other hand, levels of IL-6 transcripts were similar in preparations treated to remove macrophages or untreated,, showing that IL-6 biosynthesis by macrophages was a negligible source of IL-6. Finally, sites of IL-6 produc-

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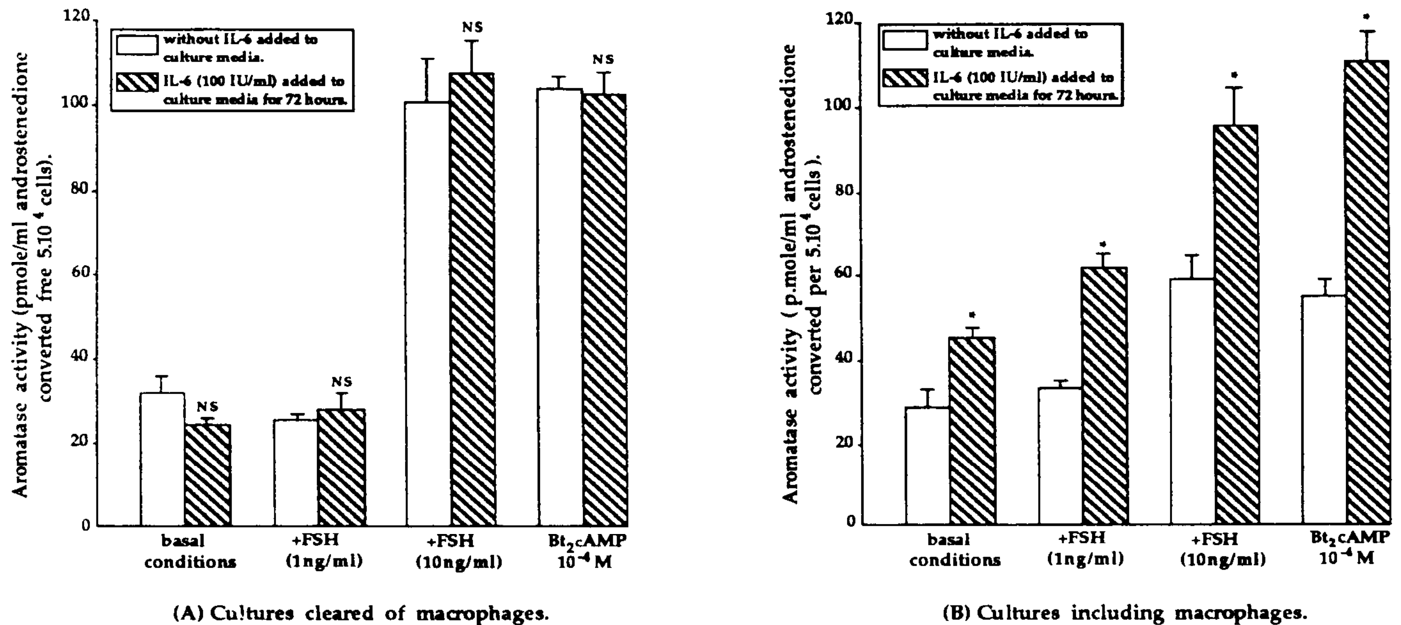

FIG. 7. Effects of IL-6 on aromatase activity in GC cultures free of macrophages (A) and in GC cultures including macrophage contaminants (B). rhIL-6 at a dose of 100 IU/mL was added for 72 h to serum-free medium supplemented with 10^{-3} mol/L testosterone in the basal state and under stimulated conditions in the presence of 1 and 10 ng/mL FSH or 10^{-4} mol/L $(Bu)_2cAMP$. Controls were completed in cultures without rhIL-6. The experiment was repeated on four series of cultures obtained from different follicular aspirates. Aromatase activity was expressed as picomoles of androstenedione converted in 1 mL culture medium over 5 h. Data are the mean ± SE. *, $P < 0.01$; NS, $P > 0.05$ (by Scheffe's test after one-way analysis of variance, *vs.* cultures without rhIL-6). Basal aromatase activity and the percent increment in aromatase activity induced by FSH or $(Bu)_2cAMP$ were not modified in cultures free of macrophages (A) with rhIL-6 *vs.* those in controls; in cultures including macrophages (B), aromatase activity was significantly increased in cultures with 100 IU/mL rhIL-6 *vs.* that in controls.

tion were clearly identified on GC-enriched preparations by positive immunostaining. Evidence of IL-6 release by GCs was previously reported (4, 18, 19). Here, characterization of IL-6 transcripts by RT-PCR, *in situ* hybridization staining, and immunostaining constitutes a data set that allowed identification of GC from the preovulatory follicle at the time of ovulation as an active site of IL-6 biosynthesis. These results provide strong support for the view that IL-6 activity detected in preovulatory follicles is derived to a large extent from a somatic ovarian cell. By establishing GC as sites of IL-6 production, our present findings provide yet another example of the variety of cellular IL-6 sites in the immune system (20–23).

IL-6 release by undifferentiated rat granulosa cells was shown to be up-regulated by FSH (4, 24) or agents that increase the cAMP cellular content of rat ovarian cells (19). Here, GCs were collected 36 h after the induction of ovulation by hCG, which is known to induce high concentrations of cellular cAMP (25), and we showed that these cells were active sites of IL-6 synthesis. Similarly, increments in the relative abundance of ovarian IL-1β transcripts after exposure to hCG has been previously noted in rats (26). Biological IL-6 activity was higher and *in situ* hybridization signals were more dense in 24-h cultures than in 72-h cultures. Here, as shown in a variety of cells, except in tumor ovarian cell lines (18), IL-6 production is probably transiently induced. The addition of hCG, which is known to maintain elevated P levels in cultures (27), did not modify IL-6 levels in terms of biological activity or *in situ* hybridization signals. Conversely, we noted that IL-6 levels decreased and E_2 levels rose when testosterone replaced LDL in serum-free cultures, suggesting that IL-6 production by GCs might be down-regulated by elevated E_2 levels. The inhibitory effect of estrogens on IL-6 production has been previously demonstrated in endometrial stromal cell cultures (28). Periovulatory steroidogenesis is characterized by a shift from E_2 production to P production after the luteinization of GCs following the LH surge. On an other hand, it has been previously noted that the lowest plasma concentrations of IL-6 were observed during the first half of the cycle, corresponding to high circulating estrogen levels, whereas the highest concentrations of IL-6 were observed just before menstruation, corresponding to low circulating estrogen levels, probably contributing to systemic alterations in body temperature during the menstrual cycle (29). It is, therefore, tempting to speculate that IL-6 might be transiently produced by GCs at the time of ovulation.

Cytokines, including IL-6, have been suggested to influence oocyte fecundity (9). Here, the presence or absence of detectable IL-6 activity in OCC supernatants did not significantly modify oocyte fertilization rates. IL-6 activity detected in OCC supernatants might originate from cumulus cells, because IL-6 activity was detected in cumulus cell cultures, whereas culture medium from denuded oocytes was devoid of IL-6 activity. In addition, we noted that IL-6 levels in cumulus cell cultures were much lower than IL-6 levels in GC cultures. Marked differences in physicochemical properties, hormonal responsiveness, and LH/hCG receptor dis-

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tribution have been notified between cumulus and granulosa cells (30, 31). The present observations might constitute one more means of differentiating the two cellular types.

Although IL-6 activity detected in FF samples was usually less than 50 IU/mL, IL-6 locally derived from activated resident follicular cells including GCs may assure, by cell to cell contact, that higher concentrations of IL-6 are delivered to target cells. A variety of IL-6-responsive cells, including endothelial cells, is present in the preovulatory follicles at ovulation. In primates, the dominant follicle is richly vascularized. After ovulation and disintegration of the basal lamina, thecal vessels invade the granulosa layer, giving rise to the luteal vascular network. It seems likely that the centrally located GC are one of the factors controlling the development of luteal blood cell vessels (32); by stimulating endothelial motility (33), IL-6 produced by GC might have an active role in angiogenesis.

The periovulatory period is marked by a rise in P biosynthesis. This steroid is secreted in large amounts by GC and plays a decisive role in embryo implantation, supporting ovarian function in the early luteal phase. With exogenous P, it has been considered a rational approach to improve rates of pregnancy in patients undergoing IVF (34). Numerous cytokines have been demonstrated to up or down-regulate P release by human GCs, whereas some cytokines, including IL-1 (35, 36), tumor necrosis factor-α (37–39), and IL-6 on porcine GC (40), have no effect on steroidogenic activities of GC. Our experimental conditions achieved conditions of low endogenous IL-6 production in GC cultures, which let us test the effects of adding increasing rhIL-6 doses. In GC cultures cleared of macrophages, IL-6 had no effect on basal and stimulated steroidogenic activities of GCs. The lack of IL-6-mediated effects might occur either with or without the loss of IL-6 receptors. Future studies will be necessary to investigate the presence of IL-6 receptors on GC. Conversely, we showed that adding rhIL-6 to GC cultures containing macrophage contaminants modulated steroidogenic activities of GCs, including P production. As IL-6 did not mediate any direct effect on GCs, the activating effects of IL-6 might be induced by the release of macrophage factor(s). Mouse luteal cells have been demonstrated to produce more P when they were recombined in cultures with autologous macrophages from corpora lutea (41), whereas macrophages enhanced P production by human cultured GCs (42). During the process of aspiration, FF might be mixed with blood, and monocytes could contaminate the follicular cell population. However, previous studies showed that only a small contamination of GCs with blood monocytes existed, and mostly monocytes were present in follicular cell cultures originated from follicular tissue (7). These findings give a novel model for cytokine-mediated relations in the human preovulatory follicle by showing that macrophages, via IL-6, might be involved in GC steroidogenesis.

Acknowledgments

We thank Dr. P. Granet (IVF-ET Laboratory, Hôpital Universitaire, Paris, France) and Dr. A. Thebault (Center for Reproductive Medecine, Hôpital Américain, Neuilly, France) for providing us with follicular aspirates.

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