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Luteal leukocytes are modulators of the steroidogenic process of human mid-luteal cells

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Flow cytometry analysis of luteal cells revealed that an important proportion of these cells are leukocytes. The percentage of leukocytes was higher in the early (42 ± 4) and late (35 ± 3) luteal phases than in the mid-luteal (24 ± 2) phase. However, the proportion of macrophages did not differ between the luteal stages. The flow cytometric properties correlated with cellular size and granularity were not reliable as discriminators of luteal cell subpopulations. Therefore, to assess the contribution of luteal leukocytes, these cells were completely removed from luteal cell suspensions (total cells), by a negative selection procedure (immunomagnetic separation). The functional role of leukocytes in mid-luteal steroidogenesis was assessed, in total as well as leukocyte-depleted cells. Progesterone production was found to have increased 2.2-fold in leukocyte-depleted cell cultures, in comparison with total cells under basal conditions. However, the response to human chorionic gonadotrophin (HCG) was 36% lower under the latter conditions. Oestradiol production was not significantly modified under basal or HCG-treated conditions. In leukocyte-depleted cells, the concentration of interleukin (IL)-1β decreased 5-fold in comparison with total cell cultures, suggesting that leukocytes are the principal source of IL-1β. In summary, the results of the present investigation suggest functional interactions between the immune system and steroidogenic cells of the human corpus luteum.

Key words: corpus luteum/flow cytometry/interleukin-1β/leukocyte/steroidogenesis

Introduction

The corpus luteum of several species is comprised of several cell types, including steroidogenic cells, vascular cells and fibroblasts. In addition, macrophages, neutrophils and lymphocytes have also been detected (Lei *et al.*, 1991; Brännström *et al.*, 1994; Bukovsky *et al.*, 1995). Leukocytes are particularly important among the non-steroidogenic cells, due to their reported regulatory effects on the steroidogenic process (Kirsh *et al.*, 1981; Yamamouchi *et al.*, 1992). On the other hand, these cells express and secrete a number of molecules that affect steroid production (Wang *et al.*, 1992; Brännström and Norman, 1993; Best and Hill, 1995). Moreover, steroid and peptide receptors have been detected in leukocytes, raising the possibility that these cells may be endocrine regulated (Grossman, 1985; Ciocca and Vargas, 1995; Sugino *et al.*, 1996).

The majority of the cellular assessments of the different human luteal cell types by morphological/immunohistochemistry analyses, have shown quantitative and qualitative cellular changes during the menstrual cycle, suggesting that these cellular variations are of physiological importance. However, limited data are available and some are contradictory (Lei *et al.*, 1991; Retamales *et al.*, 1994; Vega, *et al.*, 1994a; Best *et al.*, 1996). Although the information obtained from these studies is valuable, further investigation is needed to understand the cell–cell communication, and the physiological significance of the cellular changes in the human corpus luteum. The aim of the present investigation was to study, *in vitro*, the role of leukocytes in luteal function. Two approaches were employed: (i) immunocytometric: flow cytometry for leukocyte quantification throughout the corpus luteum life span; (ii) functional: culture of luteal cells in which the leukocytes have been removed by immunomagnetic separation. Luteal steroidogenesis was assessed in the presence and absence of leukocytes as well as under basal and human chorionic gonadotrophin (HCG)-stimulated conditions. In addition, the production of interleukin (IL)-1β, a secretory molecule of leukocytes that has been postulated as an intraovarian regulator (Brännström *et al.*, 1993; Hurwitz *et al.*, 1992, 1997), was also assessed.

Materials and methods

All chemicals, culture media, and hormones used were obtained from Sigma Chemical Co. (St Louis, MO, USA), and collagenase from Worthington Biochemical Corporation (NJ, USA). Ligand Immuno-EnzymoMetric Assay (IEMA) kit for measurement of IL-1β was obtained from Immunotech (Marseilles, France).

Antibodies

The following antibodies were obtained from generous gifts of: Dr María Rosa Bono (Faculty of Sciences, University of Chile, Santiago, Chile); monoclonal antibody (mAb) W6/32 [immunoglobulin (Ig)G_{2a} anti-human major histocompatability complex (MHC) Class I; Barnstable *et al.*, 1978], and mAb GAP8.3 (IgG_{2a} anti-CD45; Berger *et al.*, 1981) and Dr Francisco Sanchez-Madrid (The Princess Hospital, Madrid, Spain); mAb LIA 3/2 (IgG_1 anti-CD18; Campanero *et al.*, 1993), and mAb Bear-1 (IgG_1 anti-CD11b; Keizer *et al.*, 1985). The

mAb Tük4 (IgG_1 anti-CD14) were purchased from Dako (CA, USA), and the labelled $F(ab')_2$ fragment antibody (anti-mouse IgG) from Immunotech (Marseilles, France).

Patients

Corpora lutea were obtained from 16 normal women, aged 30–44 years, undergoing mini-laparotomy for tubal sterilization at the San Borja-Arriarán Clinical Hospital, Santiago, Chile. The study was approved by the Institutional Review Board and all patients gave their informed consent for removal of corpora lutea. After removal, the tissue was placed in a sterile saline solution and transported to the laboratory at room temperature. The corpora lutea were washed with saline to remove blood. The cycle-date day of each woman was confirmed by an endometrial biopsy (Noyes *et al.*, 1950) and classified as early (1–4 day; $n = 7$), mid (5–9 day; $n = 6$) or late (10–14 day; $n = 3$) luteal stages.

Cell dispersion

Luteal cells were dispersed as described earlier with minor modifications (Devoto *et al.*, 1989; Vega *et al.*, 1994a,b). Briefly, corpora lutea were enzymatically dissociated in culture medium (medium 199/HEPES 25 mM/$NaHCO_3$ 26 mmol/l and penicillin 50 IU/ml) with collagenase trypsin-free (740 IU/100 mg tissue), and DNase (14 kIU/100 mg tissue). After 90 min, luteal cells were washed once with culture medium and twice with Dulbecco's phosphate-buffered saline Ca^{2+}- and Mg^{2+}-free (PBS), containing EDTA (1 mM). Blood cells were removed from the luteal cellular suspension by means of a Ficoll–histopaque/PBS gradient 1.077 then washed three times in PBS/0.1% bovine serum albumin (BSA). The viability was >90%, as assessed by the Trypan Blue exclusion method.

Luteal leukocyte isolation

Immediately after cellular dispersion, a fraction of luteal cells was incubated with a panel of mouse mAbs (GAP 8.3, LIA 3/2 and Bear-1) directed to leukocyte specific molecules for 40 min at 4°C, washed twice, and then incubated with sheep anti-mouse IgG-immunobeads (Dynal Co.) in 100 µl of PBS/0.1% BSA (1 cell:4 beads), for 30 min at 4°C, with gentle rolling. Leukocytes were separated from luteal cells in a magnetic concentrator (Dynal Co.); three periods of 2 min with 3 ml of PBS/0.1% BSA). Viability was >90%, as assessed by the Trypan Blue exclusion method.

Culture

Luteal cells were cultured as described earlier (Devoto *et al.*, 1989; Vega *et al.*, 1994a). Briefly, total and leukocyte-depleted luteal cells were incubated in culture medium. Immediately after plating, incubations were carried out in duplicate in the absence (basal) and presence of HCG (10 IU/ml). Cultures were terminated at 24 h and the media were stored at –20°C until assayed for steroid concentration by specific radioimmunoassay (Devoto *et al.*, 1980), and for IL-1β concentration by IEMA. The assay for IL-1β was a sandwich type, using a first mAb anti-IL-1β and a second acetylcholinesterase-conjugated antibody. The intensity of the reaction with the chromogenic substrate was determined by absorbance at 410 nm; the sensitivity was 5 pg/ml. The results obtained were normalized to 10^6 cells. In addition, the basal steroid production of leukocyte-depleted luteal cells was corrected by subtraction of the increment of steroidogenic cells after leukocyte isolation.

Flow cytometry

Cells in suspension were incubated with the primary antibodies for 40 min at 4°C, in 100 µl of the sample at a concentration of 2×10^6/ml. Primary antibodies were used at saturation, as established after titration by flow cytometry. Following the incubation period, the cells were washed, resuspended in 100 µl of the fluorescence-conjugated $F(ab')_2$ fragment antibody and incubated for an additional period of 30 min at 4°C. After washing, the cells were resuspended at a concentration of 0.6×10^6/ml in PBS containing 0.5% formaldehyde and stored overnight in the dark until analysis. Fluorescence analysis was performed using a FACScan® scanner and Lysys II software (Becton Dickinson, San José, CA, USA). Flow cytometric analysis was performed using standard settings: fluorescence 1 (FL1), 4 decades (logarithmic) detector 430 V, log amplifier. These settings were selected after multiple experiments to evaluate reproducibility. For each sample, the analysis was based on measurement of 3000–20 000 nucleated cells. Control samples included an autofluorescence, an isotype-matched control, an antibody-treated and a positive control (stained with anti-MHC I mAb W6/32) for nucleated cells. Before each experiment, the cytometer was aligned and standardized with a microbead standard (Coulter Immunology, Hieleach, FL, USA). The light scattered by the cells parallel to the laser beam is proportional the the cell's cross-sectional area and hence cellular size. Conversely, the light dispersed by the cells perpendicular to the laser beam, depending upon refraction and reflection of light is proportional to the external and internal complexity of the cells (cellular granularity). The forward angle light scatter and side angle 90° light scatter correlated with cellular size and granularity respectively, were analysed using Lysys II. Previously, the total luteal cells stained with three mAb anti-leukocyte molecular markers (CD18, CD45 and CD11b) and detected using green fluorescence (FL1). The scatter properties were analysed separately for leukocytes and non-leukocyte cells using different regions or gates in FL1 for leukocytes and non-leukocyte cells (negative stain).

Statistical analysis

The data are presented as means ± SE. Data were analysed by two-tailed Student's *t*-test for the comparison between basal and treated conditions and between different luteal cellular populations. $P < 0.05$ was considered to be statistically significant.

Results

Detection of leukocytes in corpora lutea from different ages

The whole leukocyte and macrophage component of human luteal cells throughout the corpus luteum life-span was detected and quantified by flow cytometry. To improve the detection efficiency of leukocytes, a battery of mAbs directed to CD45, CD18 and CD11b were used as markers. Figure 1 indicates that the percentage of leukocytes present in total dispersed luteal cells was 20–52%, and was significantly lower in the mid-luteal phase than in the early and late phases ($P < 0.01$). On other hand, the percentage of macrophages, detected using a mAb anti-CD14, was not different at different stages of the luteal phase. In mid-luteal phase corpus luteum, the ratio of leukocytes/macrophages was close to one, indicating the presence of a significant number of macrophages during the mid-luteal phase. However, in the early and late luteal stages, other leukocyte subtypes may be present.

Scatter characteristics of luteal cells

To investigate possible differences in the distribution of cellular size and granularity between luteal cell subtypes, the scatter characteristics of leukocytes and non-leukocyte cells were

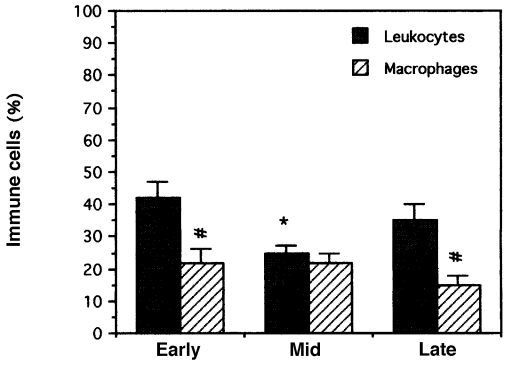

Figure 1. Flow cytometric quantification of total leukocytes and macrophages in 16 human corpora lutea from different ages. Solid bar: leukocytes percentage ± SE from early ($n = 7$), mid ($n = 6$) and late ($n = 3$) corpus luteum. Hatched bar: macrophages percentage ± SE from early ($n = 6$), mid ($n = 4$) and late ($n = 2$) corpus luteum. *Significantly different from early and late luteal phases, $P < 0.01$. #Significantly different from leukocytes in early or late luteal phase respectively, $P < 0.05$.

analysed by flow cytometry in cells from different luteal stages. Figure 2 shows a representative analysis and illustrates the forward scatter (cell size) and side scatter (granularity) distribution of leukocytes and non-leukocyte cells. For the separate assessment of leukocytes and non-leukocyte cells, a histogram of fluorescence for CD45, CD18 and CD11b markers (Figure 2A), the non-leukocyte cells were gated in region 1 (R1) and leukocytes in region 2 (R2). The histograms in Figure 2B–E illustrate the forward scatter and side scatter of leukocytes and non-leukocyte cells respectively. The results show an important overlap between both cell populations in forward scatter and side scatter, which makes the identification of these cell populations by these parameters impossible. Moreover, in non-leukocyte cells only one peak was observed (Figure 2B,D), indicating that cellular size and granularity were equally distributed, and it was not possible to discriminate between luteal cell subpopulations. However, the analysis of the distribution of these parameters in leukocytes from different luteal stages identifies two to three peaks in forward scatter and two peaks in side scatter (Figure 2C,E respectively), suggesting the presence of leukocyte subtypes. The scatter distribution of macrophages corresponded with cells of larger size and granularity (dotted lines overlapping in Figure 2C,E), indicating the presence of macrophages.

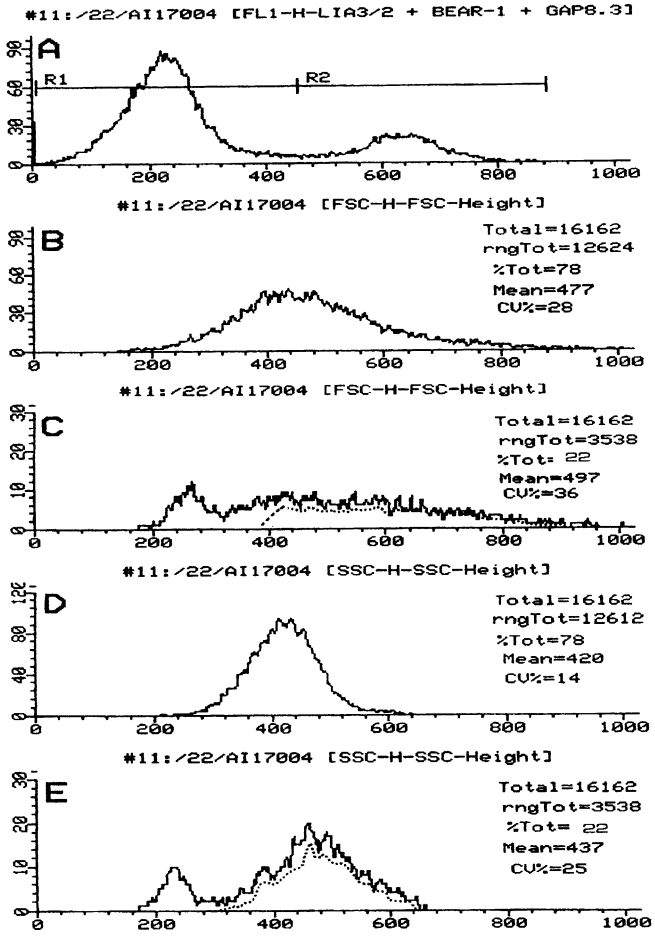

Figure 2. Flow cytometry analysis of forward scatter (FSC) and side scatter (SSC) characteristics in leukocyte and non-leukocyte populations of the mid corpus luteum. The detection of leukocytes was performed using GAP8.3 (anti-CD45), LIA3/2 (anti-CD18) and Bear-1 (anti-CD11b) monoclonal antibodies. (**A**) Histogram of fluorescence 1 (FL1) where non-leukocyte cells are delineated in region 1 (R1) and leukocytes in region 2 (R2). (**B**) Histogram of FSC in R1. (**C**) Histogram of FSC in R2, in dotted lines was overlapped the FSC-histogram of macrophages. (**D**) Histogram of SSC in R1. (**E**) Histogram of SSC in R2.

Depletion of leukocytes from suspension of total luteal cells

The cellular composition of a dispersed corpus luteum is heterogeneous, containing several types of cells and, therefore, interactions between them could be masked. To unmask leukocyte–steroidogenic luteal cell interactions, dispersed mid-luteal cells were depleted of leukocytes by negative selection, as described in the Materials and methods section, using mAbs specific to leukocyte (CD45, CD18 and CD11b) molecular markers. Figure 3 compares the histograms of fluorescence (FL1) for three leukocyte markers obtained in luteal cells, before (pre-selection) and after the leukocyte-depletion procedure (post-selection). This representation clearly shows the elimination of the leukocyte population post-selection. Similar histograms (data not shown) were obtained when the mAb anti-CD14 was used to identify macrophages, confirming its elimination after the leukocyte-depletion procedure.

Steroidogenic response of total and leukocyte-depleted mid-luteal cell population

The effect of leukocytes on the steroid production was assessed in total and leukocyte-depleted mid-luteal cell culture systems, in the absence (Figure 4A) or the presence of HCG (Figure 4B). In order to perform a valid comparison of basal steroid production between total and leukocyte-depleted cells, the data were corrected in leukocyte-depleted cells by the percentage of leukocytes during the mid-luteal phase. The leukocyte-depleted luteal cell system shows a significant increase (2.2-fold) in progesterone production, while HCG-stimulated progesterone production by leukocyte-depleted luteal cell population was decreased by 36%, in comparison with total cells. Basal oestradiol production was also increased in one leuko-

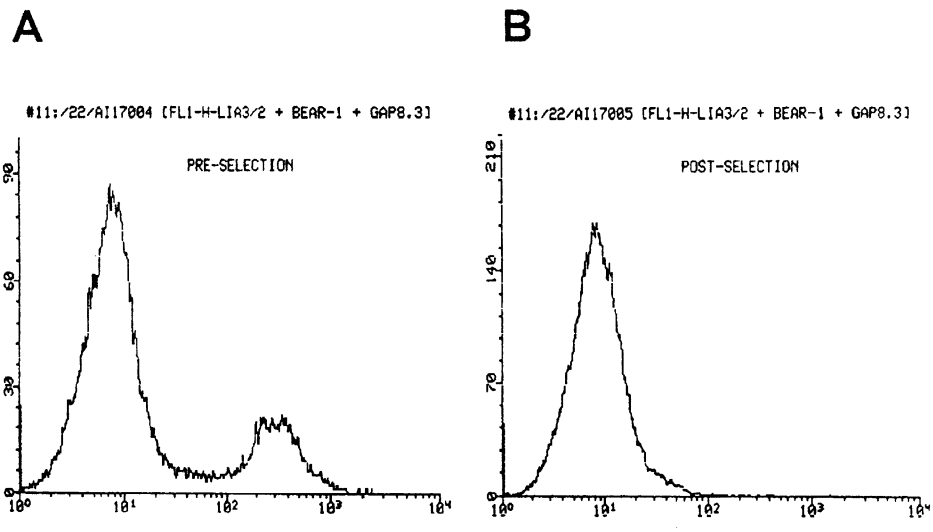

Figure 3. Flow cytometry analysis of the leukocyte depletion procedure from total cellular suspension of mid corpus luteum. Luteal cells were used for leukocyte detection, before (pre-selection) and after (post-selection) of leukocyte depletion. Cells were stained with negative control antibodies (not shown) of the corresponding isotypes, or with anti-leukocyte marker mAbs LIA3/2, GAP8.3 and Bear-1 (shown in figure) followed by fluorescein isothiocyanate (FITC)-labelled fragment $F(ab')_2$ antibody. Leukocytes (20% of the cells) were totally eliminated (<1%) after the leukocyte-depletion procedure.

cyte-depleted system, although the difference was not significant. HCG-stimulated oestradiol production was not modified.

Interleukin-1β in total and leukocyte-depleted mid-luteal cell cultures

Table I illustrates the concentration of IL-1β present in the culture media, from total and leukocyte-depleted mid-luteal cell cultures. After leukocyte deletion, the IL-1β basal concentration (986 ± 141 pg/10^6 cells) decreased ~5-fold ($P < 0.05$); no differences were found in basal compared with HCG-treated conditions.

Discussion

In the present study, we applied an immunomagnetic technique to isolate leukocytes from human total luteal cellular suspension. The use of three antibodies directed to CD18, CD45 and CD11b leukocyte molecular markers, confirmed previous information that leukocytes are an important cellular component of the human corpus luteum. It is interesting to note that the quantitative distribution of leukocytes in human corpora lutea of different ages was lower during the mid-luteal phase. The macrophage distribution did not change during the luteal stages; moreover, macrophages were the predominant leukocytes found during the mid-luteal phase. On the other hand, it is well known that the early corpus luteum is highly haemorrhagic, suggesting that blood lymphocytes are the predominant leukocytes determined in this phase. Also, it is possible that the high numbers of leukocytes present in early corpus luteum may be due to infiltration of monocytes and granulocytes during the periovulatory process (Brännström and Norman, 1993; Brännström *et al.*, 1994; Best *et al.*, 1996). Conversely, in the late corpus luteum, the increase in the percentage of leukocytes, without a change in the proportion of macrophages, may indicate immigration of T lymphocytes during the mid- to late luteal phase, as has been suggested by

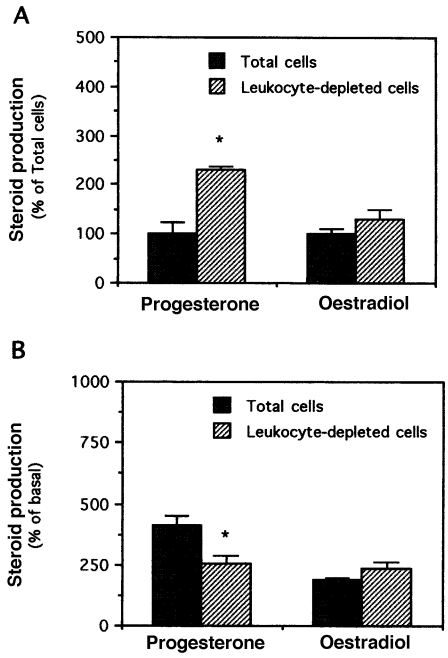

Figure 4. (**A**) Basal and (**B**) human chorionic gonadotrophin (HCG)-stimulated steroid production by leukocyte-depleted and total luteal cells of mid-luteal phase. Luteal cell populations were cultured in the presence or absence of HCG (10 IU/ml) for 24 h. Leukocytes represented almost 24% of the total luteal cells, therefore the increased number of luteal steroidogenic cells in leukocyte-depleted cells was divided by a factor of 1.3. Values represent mean ± SE from eight cultures and are representative of two independent corpora lutea. Upper panel: a significant major progesterone production was obtained in leukocyte-depleted luteal cells versus total cells, while oestradiol production was not affected (basal: progesterone 58 ± 13; oestradiol: 2.2 ± 0.2 ng/10^6 cells). Lower panel: progesterone production in response to HCG by leukocyte-depleted cell cultures was significantly different from total luteal cells, while HCG-stimulated oestradiol production was not different between both cell culture systems. *$P < 0.01$.

Table I. Cytokine interleukin (IL)-1β production by total and leukocyte-depleted mid-luteal cells. Values represent mean ± SE of triplicates of three separate mid-luteal phase corpora lutea

	Total ($pg/10^6$ cells)	Leukocyte-depleted* ($pg/10^6$ cells)
Basal	986 ± 141	172 ± 43
HCG	1115 ± 230	220 ± 87

HCG = human chorionic gonadotrophin.
*Significantly different compared with total cells, $P < 0.05$.

Best *et al.* (1996). On the other hand, the assessment of leukocytes in luteal cells showed a similar percentage of positive cells between CD18 and CD45 and CD11b, indicating that these markers were not present in cells other than leukocytes. An important consideration is the use of these antibodies in association with the immunobead technique, resulting in a complete depletion of leukocytes, as assessed by flow cytometry. Analysis of forward and side scatter of leukocytes and non-leukocyte luteal cells showed a wide range of these characteristics in non-leukocyte cells, and an important overlap was encountered between both types of cell population; indicating that these parameters could used as reliable discriminators of subpopulations of non-leukocyte cells or between leukocytes and other types of luteal cell. Studies in other species, e.g. monkey (Hild-Petito *et al.*, 1989) or non-primate species (Brannian *et al.*, 1993), have identified three luteal cell subpopulations using a two-dimensional histogram of forward and side scatter. In the present study, using similar analyses of luteal cells of different ages, we always found a predominant cellular population. Additionally, a small luteal cell population of minor size and granularity was evident, corresponding to non-macrophage leukocytes. However, the subpopulation of major granularity and size found by these authors was not found in our study. The difference between our results and those of Hild-Petito *et al.* (1989) and Brannian *et al.* (1993) may be due to species differences, and the experimental approach used to identify the immune cells.

To understand the functional role of leukocytes on the steroidogenic process, luteal cell suspensions were depleted of leukocytes, and steroid production was compared under both experimental conditions. The removal of leukocytes from the culture system leads to a significant enhancement of progesterone production over 24 h, when compared with production by total mid-luteal cells. It is interesting to note that the inhibitory effect of leukocytes did not compromise oestradiol production. The regulation of steroid synthesis by the human corpus luteum is a complex phenomenon. It is well known that luteinizing hormone (LH) and HCG are the principal modulators; however, their action is luteal age-dependent, and several molecules acting in an autocrine/paracrine manner may influence the steroid production of the different steroidogenic luteal cell subtypes. Previous studies carried out in our laboratory indicated that the addition of oestradiol to a luteal cell culture inhibited progesterone production, by reducing the 3β hydroxy steroid dehydrogenase enzyme activity (Vega *et al.*, 1994b). However, in the present study it is unlikely that oestradiol could play an inhibitory role in progesterone production, since oestradiol production did not change with or without leukocytes in the culture system, under basal or HCG-treated conditions. Morphometric techniques provided quantitative data on the cellular components of the human corpus luteum (Lei *et al.*, 1991). Moreover, the differential steroidogenic capacity of human luteal cell subpopulations has been described (Ohara *et al.*, 1987; Carrasco *et al.*, 1996). Thus, large luteal cells are the principal source of basal steroidogenic synthesis, while small luteal cells are responsible for the HCG-stimulated steroid production. Nevertheless, evidence exists which indicates that large luteal cells are desensitized *in vitro* to acute gonadotrophin stimulation, but which does not support the view that these cells are independent of LH/HCG (Hutchinson and Zeleznik, 1984; Frazer *et al.*, 1989). The fact that in the absence of leukocytes in the culture system of mid-luteal cells in basal conditions, a significant increase of progesterone production is observed, would suggest that leukocytes may inhibit progesterone production by the large steroidogenic cell subpopulation. Therefore, the inhibitory effect of leukocytes may be operating in a step not regulated by HCG, in large luteal cells, which exhibit a low number of LH/HCG receptors. On the other hand, the finding that basal oestradiol production is unchanged when leukocytes are removed, suggests that these immune cells are not affecting the basal steroid production by small cells, which has been reported as the principal source of oestradiol precursor (Ohara *et al.*, 1987).

Interestingly, progesterone production in the HCG-treated condition of leukocyte depletion was 36% lower regarding total luteal cells. In this respect, recent investigations conducted in our laboratory indicated that IGF-I acts in a synergistic manner with HCG in the enhancement of progesterone synthesis (Devoto *et al.*, 1995). The fact that IGF-I mRNA is expressed in macrophages (Rom *et al.*, 1988) could support the hypothesis that the lack of leukocytes in the HCG-treated condition could contribute to the diminished steroidogenic response of small luteal cells to HCG. It is also possible, that in the absence of leukocytes, small luteal cells exhibit their maximal steroidogenic capacity.

The cytokine IL-1β is found in significant concentrations in the human follicular fluid (Bränström *et al.*, 1993). Recent studies demonstrated that IL-1β and its receptor are expressed in human granulosa cells suggesting a role in ovarian physiology (Hurwitz *et al.*, 1992). Our experimental design of mid luteal cellular culture depleted of leukocytes indicates that leukocytes are the principal source of IL-1β in the human corpus luteum. A significantly lower concentration of IL-1β was detected in leukocyte-depleted culture media. It has been demonstrated that IL-1β stimulates prostaglandin F-2α (PGF-2α) production in bovine and primate luteal cells (Young *et al.*, 1997). Moreover, in several species PGF-2α has an antisteroidogenic effect in large luteal cells, which require functional cooperation between the different luteal cell types (Lamprecht *et al.*, 1975; Yen and Jaffe, 1991; Girsh *et al.*, 1996). Thus, it is possible that IL-1β or other substance(s) produced by leukocytes, may mediate the negative leukocyte effect. In agreement with the former, it has been recently reported in the rat that IL-1β may be mediating pleiotrophic

responses on theca–interstitial cell compartments, suggesting a paracrine/autocrine function for IL-1β in ovulation and corpus luteum formation (Hurwitz *et al.*, 1997). In additon, the production of IL-1β mainly by luteal leukocytes, as has been reported in the present study, together with the reported leukocytic infiltration during ovulation (Brännström and Norman, 1993), suggest an important role of these cells and their secretory molecules in the formation and regression of the corpus luteum.

In summary, these data indicate a functional interaction between the immune and endocrine system in the human corpus luteum. The inhibitory role of luteal leukocytes on luteal steroidogenesis suggests a functional role for these cells in the reproductive process, leading to a better understanding of the endocrine regulation of corpus luteum physiology, as well as in pathological conditions such as in endometriosis.

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