FERTILITY AND STERILITY
Vol. 53, No. 2, February 1990

Printed on acid-free paper in U.S.A.

Immunosuppressive properties of human follicular fluid

José A. Castilla, M.D.*
Ramón Molina, M.D.†‡
Miguel Angel Lopez-Nevot, M.D.*
Francisco Vergara, M.D.†
Federico Garrido, M.D.*
Alfonso J. Herruzo, M.D.†

Ciudad Sanitaria Virgen de las Nieves, Granada, Spain

Human preovulatory follicular fluids (FF) obtained in the course of stimulated cycles were analyzed for their possible immunologic functions. Different concentrations of FF (20%, 2%, 1%) inhibited the mitogenic response of normal human lymphocytes to concanavalin A (Con A). Lymphocytes were assessed for immunosuppressor activity after preincubation with FF. Lymphocyte mitogenic response to Con A was only suppressed by cells preincubated with FF at concentrations of 2% and 1% for at least 48 hours. No evidence of suppressor cell induction was seen following incubation of lymphocytes with 20% FF, nor was any significant relationship between FF immunosuppressor activity and the outcome of in vitro fertilization observed. We conclude that some factor(s) in FF may be capable of directly inhibiting lymphocyte response and inducing immunosuppressor cell activity in vitro. Fertil Steril 53:271, 1990

Various studies have suggested a relationship between the immune system and ovarian physiology,[1–7] and many components of follicular fluids (FF), e.g., steroids, prostaglandins, histamine, have demonstrated immunoregulatory effects in vitro and in vivo.[8–11]

The purpose of the present study was to elucidate the possible immunologic functions of FF. The effects of FF, obtained from women undergoing laparoscopic oocyte harvesting for in vitro fertilization (IVF), were studied on lymphocyte proliferative response to concanavalin A (Con A). The results indicate that FF inhibits the mitogenic response of lymphocytes to Con A, and furthermore induces immunosuppressor cells in vitro.

Received February 9, 1989; revised and accepted September 26, 1989.

* Servicio Análisis Clínicos.
† Unidad de Reproducción Humana, Departamento de Obstetricia y Ginecología.
‡ Reprint requests: Ramón Molina, Unidad de Reproducción Humana, Departamento de Obstetricia y Ginecología, Centro Materno Infantil, Ciudad Sanitaria Virgen de las Nieves, Avda de la Constitución, 18014 Granada, Spain.

MATERIALS AND METHODS

Nine samples of nonbloody FF were obtained by aspiration during laparoscopy from four normal menstruating women with tubal infertility, treated with ovarian stimulation in an IVF program.[12] At the same time serum was obtained from these women. Midcycle nonpregnant serum was used as a control. The FF and serum were stored at −20°C until testing. On the day of study, FF and serum were inactivated by heating at 56°C for 30 minutes.

Induction of multiple ovulation for IVF, monitoring technique, ovum pickup, and laboratory procedures were as described previously.[12,13] Oocytes were graded as mature, intermediate, or immature according to oocyte maturity and the degree of expansion of the corona-cumulus complex.[14]

Preparation of Lymphocytes

Lymphocytes were obtained from heparinized venous blood from normal women by Ficoll-Hypaque gradient sedimentation. After washing, the cells were diluted to a concentration of 10^6 cells/mL in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY) supplemented

with 10% fetal calf serum (Flow Laboratories, Irvine, UK) and antibiotics.

Viability of Lymphocytes

The viability of lymphocytes cocultured with FF was usually >90% as assessed by eosin exclusion.[15]

Lymphocyte Proliferative Response

Aliquots of 0.2 mL of the lymphocyte suspension were incubated in triplicate in microtiter plates with Con A (1 μg/mL; Sigma Chemical Co., St. Louis, MO) and FF (20%, 2%, 1%) for 3 days in a humidified atmosphere of 5% CO_2 and 95% air. For the last 24 hours of incubation 1 μCi of ^{3}H-thymidine (specific activity 1 Ci/mmol; Amersham International, Amersham, UK) was added to each well. Proliferation was assessed by the incorporation of ^{3}H-thymidine as measured in a liquid scintillation counter and expressed in counts per minute (cpm) ± standard deviation (SD). Percent suppression compared with control cultures (lymphocytes + Con A but without FF) was calculated according to the formula:

$$\% \text{ Suppression} = \left(1 - \frac{\text{cpm in cultures with FF}}{\text{cpm in control cultures}}\right) \times 100$$

Preincubation of Lymphocytes

One milliliter of the lymphocyte suspension was incubated in plastic tubes for varying time intervals (0, 24, 48, 72 hours) with one of the following: (1) 20 μg/mL Con A (a dose found to be optimal for generating suppressor cells[16]); (2) control serum at concentrations of 20%, 2%, and 1% (vol/vol); (3) serum from women undergoing laparoscopic oocyte harvesting for IVF (20%, 2%, 1%); (4) FF (20%, 2%, 1%); or (5) alone (control cells). After culturing, the cells were washed three times with 30 mM α-methyl mannoside (Sigma Chemical Co., St. Louis, MO), and subsequently treated with 50 μg/mL of mitomycin (Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37°C, washed three times and suspended in culture medium at a concentration of 10^6 cells/mL.

Assay for Suppressor Activity of Cells Preincubated

Fresh responder lymphocytes to be used in the assay cultures were obtained from the same normal individuals who had originally provided the preincubated cells. Lymphocytes used as responder cells were prepared exactly as described above and suspended in RPMI-1640 culture medium at a concentration of 10^6 cells/mL. A 0.1 mL aliquot of responder cells was then mixed with an equal volume of cells preincubated on microtest plates. Immediately after mixing the cells, Con A (1 μg/mL) was added to each of the wells. The cultures were then incubated for 3 days, at the end of which proliferation was assessed as described above. Percentage suppression as compared with control cultures (fresh lymphocytes + Con A + lymphocytes preincubated alone) was calculated according to the formula: % suppression = [1 − (cpm of cultures containing lymphocytes preincubated with different substances (Con A, serum or FF)/cpm of control cultures)] × 100. Statistical significance was verified with Student's *t*-test or by one-way analysis of variance.

Table 1 Effect of FF on Lymphocyte Proliferation Response to Con A (1 μg/mL)

FF concentration	^{3}H-Thymidine incorporation	
	cpm[a]	Percent suppression[b]
vol/vol		
No FF added	31,462 ± 17,975	
20%	17,473 ± 14,420	53.1 ± 17.7
2%	15,510 ± 7,524	45.2 ± 18.0
1%	18,988 ± 18,668	44.5 ± 28.8

[a] Lymphocyte proliferative response expressed as mean incorporation of ^{3}H-thymidine in counts per minute ± SD. Values obtained from nine different FF.

[b] Percentage suppression was calculated as described in the Materials and Methods section (mean ± SD).

RESULTS

The lymphocyte proliferative response to Con A was inhibited by the addition of FF at concentrations of 20%, 2%, and 1% (Table 1). Lymphocytes were assessed for suppressor activity 0, 24, 48, and 72 hours after preincubation with FF. Lymphocyte mitogenic response to Con A was only suppressed by cells preincubated with FF at concentrations of 2% or 1% for at least 48 hours ($P < 0.05$). However, lymphocytes incubated with FF at a final concentration of 20% for different time intervals had no effect on this response (Table 2).

As can be seen in Table 3, lymphocyte prolifera-

Table 2 Percentage Suppression of Lymphocyte Proliferative Response to Con A (1 μg/mL) by Lymphocyte Preincubated for Different Time Intervals With FF

FF concentrations used in preincubation	Hours of preincubation			
	0	24	48	72
vol/vol				
20%	4.5 ± 2.5[a]	9.3 ± 5.1	6.5 ± 3.5	4.1 ± 4.3
2%	2.5 ± 2.8	6.7 ± 4.9	28.0 ± 4.1[b]	22.5 ± 5.6[b]
1%	4.8 ± 3.6	7.7 ± 5.0	27.5 ± 7.4[b]	25.3 ± 5.1[b]

[a] Results are expressed as mean of percentage suppression of lymphocyte proliferation response to Con A ± SD (four FF). The percentage of suppression was calculated as described in the Materials and Methods section.

[b] $P < 0.05$; versus 0 hours of preincubation.

tive response to mitogens was unaffected by lymphocytes incubated for 48 hours with different concentrations of midcycle nonpregnant serum (control serum) or serum from women undergoing laparoscopic oocyte harvesting for IVF. Concanavalin A-induced suppressor cells were used as a positive control for suppression.

All FF were obtained from follicles that contained a mature oocyte. Similar percentage suppressions of lymphocyte mitogenic response to Con A were observed with FF from follicles of which the ova were eventually fertilized versus not fertilized (Table 4).

DISCUSSION

Human FF obtained from stimulated cycles showed marked suppression of the unrelated adult lymphocyte response to Con A, implying that FF contains factors able to depress cell-mediated immune response. These observations are in agreement with those of Van Vlasselaer and Vandeputte[1] on the immunosuppressor effect of supernatants from rat ovarian cell cultures.

The immunosuppressive action of FF was not due to cytotoxicity, since cell survival was not affected. One possible explanation for this is that FF may exert its putative immunosuppressive effects by inducing immunosuppressor cells. These cells were described in the early 1970s by Gershon and Kondo,[17] who showed that specific immunological unresponsiveness to sheep red blood cells in mice could be passively transferred with lymphocytes. Our data indicate that lymphocyte proliferative response to Con A was inhibited by cells preincubated with FF at low concentrations (1%, 2%).

Bukovsky et al.[18] have suggested that immune cells play an important role during development of the rat ovarian follicle. Hill et al.[5] have reported a preponderance of $CD8^+$ (suppressor/cytotoxic) lymphocytes in human FF. It is possible that the factor(s) triggering the recruitment of $CD8^+$ lymphocytes in the ovary may be the same as those responsible for the induction of suppressor cells in vitro by human FF. Hill and coworkers[5] suggested that estrogen could stimulate proliferation and differentiation of $CD8^+$ lymphocytes in the ovary, as estradiol (E_2) receptors have recently been demonstrated on $CD8^+$ T cells.[19] However, several authors have shown that E_2 significantly inhibits sup-

Table 3 Percentage Suppression of Lymphocyte Proliferative Response to Con A (1 μg/mL) by Lymphocytes Preincubated for 48 Hours With Different Substances

Lymphocytes preincubated with	No. of experiments	Concentrations used in preincubation			
		20 μg/mL	20%	2%	1%
Con A	4	67.8 ± 3.6[a]			
Control serum[b]	4		15.5 ± 6.4	9.3 ± 1.5	13.0 ± 6.9
IVF serum[c]	4		24.8 ± 13.9	13.8 ± 20.3	23.8 ± 8.8
FF	9		24.0 ± 11.8	35.7 ± 7.8[d]	34.7 ± 9.3[d]

[a] Results are expressed as mean percentage suppression of lymphocyte proliferative response to Con A ± SD. The percentage of suppression was calculated as described in the Materials and Methods section.

[b] Control serum was midcycle nonpregnant serum.

[c] IVF-serum was from the same women who provided FF.

[d] $P < 0.01$; versus control serum at the same concentration.

Table 4 Relationship Between Percentage Suppression of Lymphocyte Proliferative Response to Con A (1 μg/mL) by FF and the Outcome of IVF

FF concentration	Fertilized oocytes (n = 5)[a]	Unfertilized oocytes (n = 4)	*P*
vol/vol			
20%	16,540 ± 14,709[b] (52.7 ± 16.8)[d]	18,638 ± 16,209[c] (53.6 ± 21.4)	NS[e]
2%	14,510 ± 6,777 (44.8 ± 20.2)	16,759 ± 9,272 (45.8 ± 17.8)	NS
1%	17,842 ± 17,311 (43.9 ± 25.0)	20,422 ± 22,910 (45.2 ± 37.1)	NS

[a] n = number of different FF.
[b] Lymphocyte proliferative response expressed as mean incorporation of ^{3}H-thymidine in cpm ± standard deviation (SD) in the presence of FF from fertilized oocytes.
[c] Lymphocyte proliferative response in the presence of FF from unfertilized oocytes.
[d] Numbers in parentheses refer to the mean percentage suppression ± SD.
[e] NS, not significant.

pressor cell activity.[20–22] Other factors in FF such as prostaglandins,[9] histamine,[11] and human chorionic gonadotropin,[23] are known to induce suppressor cell.

Lymphocyte mitogenic response to Con A was inhibited by the addition of FF at a concentration of 20% (Table 1). When lymphocytes were incubated with the high concentration of FF (20%), no significant suppressor cells activity was obtained (Tables 2 and 3). Taken together, these findings indicate that FF contains factor(s) that directly inhibit the immune response and prevent the generation of suppressor cells in vitro by other factor(s). Progesterone, at concentrations that inhibit both T cell proliferation and cytotoxic cell induction, is also highly effective in inhibiting the generation of suppressor cells.[21]

Our data reveal no significant relationship between FF immunosuppressive activity and the outcome of IVF (Table 4), which supports the view that oocyte in vitro fertility is independent of the immune properties of FF.[6] The clinical relevance of these assays therefore seems limited. On the other hand, the difference between serum and FF immunosuppressive activity (Table 3) may reflect the different concentrations and/or production of immunosuppressive factor(s) by proliferating follicle cells.

Our findings suggest that FF may be critical in the interaction between the ovary and the immune system, and provide a preliminary basis for studying the potential role of FF in local ovarian immunoregulation.

Acknowledgments. We thank Ms. Karen Shashok for revising the English style of the manuscript.

REFERENCES

1. Van Vlasselaer P, Vandeputte M: Suppression of cellular immune reactions by rat ovarian cells. Transplantation 39: 541, 1985
2. Coulam CB, Ryan RJ: Premature menopause. I. Etiology. Am J Obstet Gynecol 133:639, 1979
3. Taguchi O, Nishizuka Y: Autoimmune oophoritis in thymectomized mice: T cell requirement in adoptive cell transfer. Clin Exp Immunol 42:324, 1980
4. Herriot DM, Warnes GM, Kerin JF: Pregnancy-related chemotactic activity of human follicular fluid. Fertil Steril 45:196, 1986
5. Hill JA, Barbieri RL, Anderson DJ: Detection of T8 (suppressor/cytotoxic) lymphocytes in human ovarian follicular fluid. Fertil Steril 47:114, 1987
6. Droesch K, Fulgham DL, Liu HC, Rosenwaks Z, Alexander NJ: Distribution of T cell subsets in follicular fluid. Fertil Steril 50:618, 1988
7. Rabinowe SL, Ravnikar VA, Dib SA, George KL, Dluhy RG: Premature menopause: monoclonal antibody defined T lymphocyte abnormalities and antiovarian antibodies. Fertil Steril 51:450, 1989
8. Grossman CJ: Interactions between the gonadal steroids and the immune system. Science 227:257, 1985
9. Fischer A, Durandy A, Griscelli C: Role of prostaglandin E_2 in the induction of nonspecific T lymphocyte suppressor activity. J Immunol 126:1452, 1981
10. Polan ML, Daniele A, Kuo A: Gonadal steroids modulate human monocyte interleukin-1 (IL-1) activity. Fertil Steril 49:964, 1988
11. Rocklin RE, Haberek-Davidson A: Histamine activates suppressor cells in vitro using a coculture technique. J Clin Immunol 1:73, 1981
12. Molina R, Vergara F, Castilla JA, Garcia G, Suarez F, Esteva G, Mas M, Ramos J, Herruzo AJ: The in vitro fertilization and embryo transfer program at the "C.M.I. Virgen de las Nieves" in Granada, Spain. J In Vitro Fert Embryo Transfer 5:307, 1988
13. Molina R, Castilla JA, Vergara F, Perez M, Garrido F, Herruzo AJ: Luteal cytoplasmic estradiol and progesterone receptors in human endometrium: in vitro fertilization and normal cycles. Fertil Steril 51:976, 1989
14. Laufer N, DeCherney AH, Haseltine FP, Polan ML, Tarlatzis BC, Dlugi AM, Naftolin F: Human in vitro fertilization employing individualized ovulation induction by human menopausal gonadotropins. J In Vitro Fert Embryo Transfer 1:56, 1984
15. Castilla JA, Rueda R, Vargas ML, Gonzalez-Gomez F, Garcia-Olivares E. Decreased levels of circulating $CD4^+$ T lymphocytes during normal human pregnancy. J Reprod Immunol 15:103, 1989
16. Fineman SM, Mudawwar FB, Geha RS: Characteristics

and mechanisms of action of the concanavalin A-activated suppressor cell in man. Cell Immunol 45:120, 1979
17. Gershon RK, Kondo K: Cell interactions in the induction of tolerance. Immunol 18:723, 1970
18. Bukovsky A, Presl J, Holub M: The ovarian follicle as a model for the cell-mediated control of tissue growth. Cell Tissue Res 236:717, 1984
19. Cohen JH, Danel L, Cordier G, Saez S, Revillard JP: Sex steroid receptors in peripheral T cells: absence of androgen receptors and restriction of estrogen receptors to OKT8-positive cells. J Immunol 131:2767, 1983
20. Paavonen T, Andersson LC, Adlercreutz H: Sex hormone regulation of in vitro immune response. Estradiol enhances human B cell maturation via inhibition of suppressor T cells in pokeweed mitogen-stimulated cultures. J Exp Med 154:1935, 1981
21. O'Hearn M, Stites D: Inhibition of murine suppressor cell function by progesterone. Cell Immunol 76:340, 1983
22. Ahmed SA, Dauphinée MJ, Talal N: Effects of short-term administration of sex hormones on normal and autoimmune mice. J Immunol 134:204, 1985
23. Fuchs T, Hammarström L, Smith CI, Brundin J: In vitro induction of murine suppressor T-cells by human chorionic gonadotropin. Acta Obstet Gynecol Scand 59:355, 1980