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The relationship between in vitro fertilization and naturally occurring antibodies: evidence for increased production of antiphospholipid autoantibodies*

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Objective: Assessment of possible effects of ovarian stimulation during in vitro fertilization (IVF) treatment cycles on circulating levels of antiphospholipid and antinuclear autoantibodies.

Design: The study was performed prospectively. Sera were obtained at three time points along IVF treatment cycle. Levels of autoantibodies directed against nuclear components, mitochondrial antigens, and phospholipids were determined using enzyme-linked immunosorbent assay.

Patients: Thirty-five patients, who underwent at least one previous IVF attempt, and 36 age- and sex-matched controls were analyzed. All participants were randomly selected.

Results: The mean levels of antiphospholipid (but not antinuclear) autoantibodies in sera from IVF-treated patients were found to be significantly higher than the corresponding values of the control group (for immunoglobulin [Ig]M isotype: anticardiolipin, antiphosphatidyl L-serine; for IgG isotype: anticardiolipin, antiphosphatidyl L-serine, and antiphosphatidylcholine; $P < 0.0001$, assessed by Mann-Whitney test). The autoantibody levels remained more or less constant at different time points along the treatment cycle. No correlation with age and number of previous IVF cycles was demonstrated.

Conclusions: Serum levels of antiphospholipid (but not antinuclear) autoantibodies increase after IVF treatment. Based on these preliminary data, it is not yet possible to estimate if the observed changes in autoantibody levels might have any future clinical influence on infertile patients undergoing IVF treatment. Fertil Steril 56:718, 1991

Differences in immune response between females and males are well recognized. Females have higher frequency of autoimmune diseases, delayed graft rejection, and higher antibody production after immunization.[1,2] This sex-related dichotomy in immune capabilities is thought to arise from the modulation in vivo of immune functions by sex hormones. Estrogens have been shown to regulate the synthesis of immunoglobulins (Igs), including IgM, IgA, and IgG,[3] to enhance production of antibodies to a variety of thymic-independent antigens and autoantigens,[4,5] and to influence the onset, development, and course of autoimmune diseases.[1,2] It

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is generally believed that sex hormones induce their effect on target cells by interacting with specific steroid receptors. Apart from the reproductive system, such receptors have been found in various tissues, including the immune system in which it appears that T-cells are the primary target of sex hormone action.[6] In humans, estrogen (E) receptors have been demonstrated in peripheral $CD8^+$ T-cells that have suppressor/cytotoxic activity. However, a relationship has also been suggested between Es and $CD5^+$ B-cells, which are thought to be involved in autoimmune phenomena.[7] Recently, it has been observed that E can induce increased production of naturally occurring antibodies in normal mice by augmenting the activity of $CD5^+$ B-cells.[8]

During in vitro fertilization (IVF) treatment, because of multiple follicular development, circulating estradiol (E_2) can reach levels that are ten times higher than in natural, nonstimulated cycles. The immediate complications of this treatment are well documented.[9,10] However, no comprehensive surveys have been performed concerning its long-term hazards. The aim of the present study was to investigate the effect of the extreme hormonal fluctuations caused by exogenously administered gonadotropins on autoantibody production in infertile patients undergoing IVF treatment.

MATERIALS AND METHODS

Patients

The study group consisted of 35 women undergoing an IVF treatment cycle in the IVF Unit at the Beilinson Medical Center. All were ovulating regularly, had normal endocrine profiles, and had undergone at least one IVF cycle in the past. Thirty-six age-matched menstruating women, subdivided into three separate subgroups, were selected as controls. They included: (group a) 15 women who have not conceived and did not receive hormonal treatment including any kind of oral contraceptive (OC); (group b) 10 nulligravid women who were treated by OC at the time of or previous to the study; and (group c) 11 women who previously delivered infants. Because results obtained separately of groups a, b, and c did not differ significantly (data not shown), they were combined into one control group. General data characterizing these patients are presented in Table 1. All donors gave informed consent to participate in this study that was approved by the local Helsinki Committee.

Table 1 General Data Concerning the Study Population

Group	Age	Previous pregnancies	Previous IVF cycles
	y		
IVF (n = 30)	31.1 ± 9.7 (25 to 42)[a]	1.5 ± 1.2 (0 to 4)	2.76 ± 2.1 (1 to 8)
Control a (n = 15)	23.6 ± 3.9 (19 to 35)	—	—
Control b (n = 10)	27.1 ± 5.7 (21 to 40)	—	—
Control c (n = 11)	32.1 ± 5.7 (23 to 42)	2.0 ± 1.3 (1 to 5)	—

[a] Values are means ± SD with ranges in parentheses.

Superovulation Protocols

Ovarian stimulation was achieved using a combined regimen of gonadotropin-releasing hormone analog and human menopausal gonadotropin (hMG). Gonadotropin-releasing hormone analog (Superfact; Hoechst AG, Frankfurt am Main, Germany) was administered transnasally (900 μg/d) starting on cycle day 2. It was given daily until the stage of human chorionic gonadotropin (hCG) injection. Human menopausal gonadotropin (Pergonal; Teva Industries, Jerusalem, Israel) was given from the 4th day of the cycle (3 ampules; 225 IU follicle-stimulating hormone/225 IU luteinizing hormone) using an individualized protocol, adjusted according to the patient's response. Ovarian response was monitored both ultrasonically and hormonally (radioimmunoassay determination of circulating E_2 levels), and hMG treatment was stopped when E_2 levels reached 1,000 pg/mL. Ovulation was induced by hCG, which was administered when at least two preovulatory follicles were detected.

Serum Samples

Patient's blood samples were drawn at three time points: T_1, before commencing treatment (days 1 to 2 of the menstrual cycle); T_2, at the time of the expected E_2 peak (days 9 to 11), before administering hCG; and T_3, 14 days after oocyte retrieval. Control samples were taken during the early follicular phase (days 1 to 3 of a spontaneous, nonstimulated, menstrual cycle). Serum was collected from 2-mL nonheparinized blood samples and stored frozen until use.

Determination of Circulating Autoantibody Levels

Levels of autoantibodies directed against the following antigens were assessed: (1) nuclear compo-

nents (Smith antigen; small nuclear ribonucleoprotein [RNP]; Sjögren's syndrome-related antigen [SS-B/La]; double-stranded deoxyribonucleic acid [DNA]; single-stranded DNA; deoxyribonucleoprotein [DNP]); (2) M-2 mitochondrial antigen (pyruvate dehydrogenase); and (3) phospholipids (cardiolipin; phosphatidylcholine; phosphatidyl L-serine). Detection was performed using enzyme-linked immunosorbent assay (ELISA) kits purchased from BioHyTech (Ramat Gan, Israel) and performed according to the manufacturer's instructions, as described elsewhere.[11,12] Briefly, the antigens were each coated optimally to wells of Nunc (Roskilde, Denmark) polystyrene microtiter plates. Serum samples were diluted 1:200 in assay diluent, added to the wells, and incubated for 30 minutes at 37°C. After washing the plates with phosphate-buffered saline (PBS)-Tween 20 wash buffer, (phospholipid wash buffer did not contain Tween), a trivalent conjugate containing a mixture of alkaline phosphatase-conjugated goat antihuman IgG, IgM, and IgA (each optimally diluted for each particular antigen, dilution range of 1:1,000 to 1:3,000) was added to the wells, and plates were again incubated for 30 minutes at 37°C. The plates were again washed, and diethanolamine substrate buffer containing P-nitrophenyl-phosphate and $MgCl_2$ was added. After 60 minutes at 37°C, the optical density (OD) in each well at 405 nm was read with Titertek ELISA reader (Lab System and Flow, Irvine, United Kingdom). Standards provided in BioHyTech kits or known positive and negative sera, as well as a reagent blank, were included in each plate. Where autoantibody isotypes were assessed (versus phospholipids and single-stranded DNA), assays were performed as described above except after binding of sera plates were incubated separately with anti-IgM or anti-IgG conjugates. Optical densities were converted to arbitrary autoantibody units per mL (U/mL) by comparison with the dilution curve of a high-titered positive serum included in each plate. When testing autoantibody levels against phosphatidylcholine and phosphatidyl L-serine, a standard serum was not available. Therefore, a known positive and negative sera were run as test controls, and results are presented as raw OD values. Cutoff values for positivity, which represent the mean +3 SD of antibody levels (U/mL) obtained from 210 blood bank control sera, were provided by BioHyTech. Autoantibody activities in these sera showed normal distribution (data not shown). All experimental sera with autoantibody levels higher than the cutoff value were considered positive.

Statistical Analysis

Because the data were not Gaucian in distribution, the Mann-Whitney test was used to assess differences between the groups. A P value of <0.05 was regarded as significant. The correlation between autoantibody levels and patients' age as well as number of previous IVF cycles was calculated using regression analysis. The statistical analysis was supervised by the Department of Statistical Counselling, Tel Aviv University.

RESULTS

Based on the observations that autoantibody production can be enhanced by estrogens,[8] we investigated whether extreme hormonal fluctuations caused by exogenously administered gonadotropins in infertile patients undergoing IVF treatment have similar effects. Autoantibodies directed against two major groups of antigens were assessed: (1) nuclear and mitochondrial components because of their association with autoimmune diseases and (2) phospholipids because of the suggested association between raised serum levels of naturally occurring autoantibodies to anionic phospholipids and habitual abortion.

Autoantibodies to Nuclear and Mitochondrial Components

The levels of autoantibodies directed against various nuclear antigens and against M-2 mitochondrial antigen were assessed. The patients' group included women who had undergone at least one IVF cycle before the study, and the control group was comprised of all three control subgroups specified above (see Materials and Methods). No difference could be observed between the study and the control groups in autoantibody levels directed against any of the antigens tested (Fig. 1). Likewise, the proportion of autoantibody-positive individuals was similar in the two groups (data not shown).

Autoantibodies to Phospholipids

Levels of autoantibodies directed against cardiolipin, phosphatidyl L-serine, and phosphatidylcholine of the IgM and IgG isotypes were assessed. Results obtained during the early follicular phase from

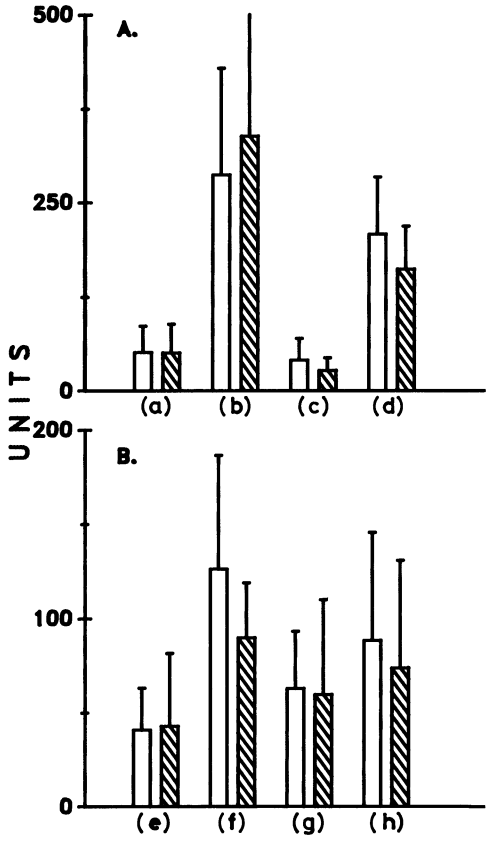

Figure 1 Levels of autoantibodies directed against nuclear and mitochondrial antigens in sera from patients undergoing IVF treatment and age-matched healthy women. All women were tested during the early follicular phase. Histograms (□, IVF group; ▨, control group) represent the means ± SD of arbitrary units (U/mL) of all the individuals included in each group, calculated in reference to a known high titered positive serum. *Panel A:* Autoantibodies directed against: (*a*) single-stranded DNA IgG; (*b*) single-stranded DNA IgM; (*c*) double-stranded DNA; and (*d*) pyruvate dehydrogenase. *Panel B:* Autoantibodies directed against: (*e*) Smith antigen; (*f*) DNP; (*g*) SS-B/La; and (*h*) small nuclear RNP.

all women included in the study are presented in Figure 2 and Table 2. In spite of the extreme variation within each group (Fig. 2), the mean autoantibody levels (Table 2) in sera from IVF-treated patients were found to be significantly higher than the corresponding values of the control group for all antiphospholipid antibodies tested ($P < 0.0001$; Mann-Whitney test), except for those directed against phosphatidylcholine of the IgM isotype. It should be emphasized that the observed augmentation of antiphospholipid antibody levels did not reflect a general enhancement of Ig production because levels of both types of anti-single-stranded DNA antibodies tested (IgM and IgG) were found to be similar in the study and control groups (Fig. 1).

One of the factors that might have contributed to the rise in autoantibody levels of IVF-treated patients is extreme fluctuations in E_2 levels during the IVF cycle. To test this possibility, we have performed the assay at three time points: at the early follicular phase, at the time of peak E_2 concentrations (before hCG administration) and 2 weeks after oocyte collection. Figure 3 summarizes the levels of anticardiolipin antibodies; similar results were obtained for the other phospholipids tested (data not shown). No difference could be detected between these three time points and their corresponding E_2 levels (data not shown), suggesting that acute short-term changes in circulating E_2 are not associated with an abrupt production of antiphospholipid autoantibodies.

In view of the lack of immediate effect of increased E_2 concentrations on autoantibody levels and because patients in the study group varied in their previous exposure to episodes of excessive rise in E_2 levels, a correlation between the number of previous

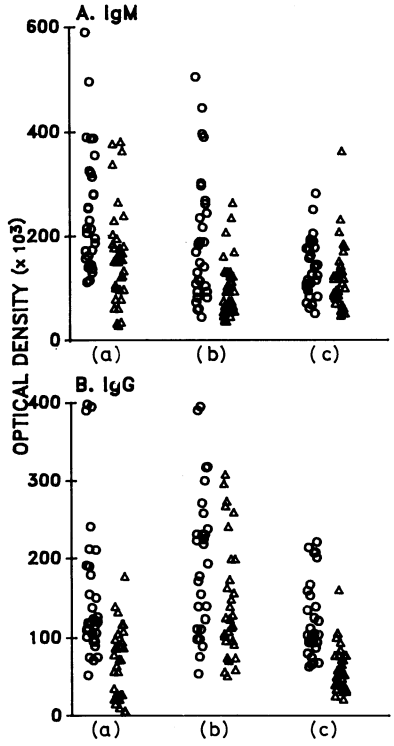

Figure 2 Scattergram of antiphospholipid autoantibody levels in sera from patients undergoing IVF treatment and age-matched healthy women. Symbols represent the OD obtained in an ELISA assay (○, IVF patients; △, controls). Autoantibodies directed against: (*a*) cardiolipin; (*b*) phosphatidyl L-serine; and (*c*) phosphatidylcholine.

attempted IVF cycles and autoantibody levels was looked for. However, no such relationship was observed except for autoantibodies directed against phosphatidyl L-serine of the IgG isotype ($P = 0.02$) (data not shown).

There are data suggesting that autoantibody levels decline with age.[13,14] We could not show any correlation of our results to age neither in the study group (data not shown) nor in the control group (data not shown), probably because of the narrow age distribution of the individuals recruited for this study (age range of 25 to 42 years).

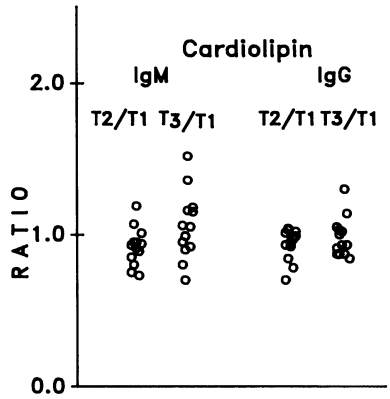

Figure 3 Ratios between anticardiolipin antibody levels at different time points along the IVF treatment cycle and baseline levels (early follicular phase, T_1). T_2, time of peak E_2 level; T_3, 2 weeks after oocyte retrieval.

DISCUSSION

The present study demonstrates that serum levels of antiphospholipid autoantibodies increase after IVF treatment, whereas those of antinuclear autoantibodies remain basically unchanged. The precise timing, in relation to the extreme hormonal fluctuation, at which this phenomenon occurs is not clear. These results may also implicate that a mere single episode of high magnitude E increase suffices to bring about this immune response. However, further experiments, involving pretreatment and long-term post-treatment assessment of each individual woman are necessary to confirm this assumption. Moreover, an effect of ovarian tissue damage induced at the time of follicular aspiration on levels of antiphospholipid autoantibodies cannot be excluded. Therefore, parallel studies are currently being undertaken in our department on patients treated solely by ovulation-induction therapy.

There is increasing evidence indicating that serum-derived antiphospholipid autoantibodies and anti-DNA autoantibodies are largely separate populations. Both types of antibodies have been shown to bind to their respective ligands with high affinity, to rarely cross-react,[15] and to differ in their IgG subclasses and light chain profiles.[16] It has also been reported that in patients with repeated abortions, those who demonstrated antinuclear antibodies appeared to be a separate clinical subgroup from those with antibodies to cardiolipin.[17] Thus, our finding that levels of anti-DNA autoantibodies remain basically unchanged in the presence of significantly raised antiphospholipid autoantibody levels is consistent with the concept that these two antibody populations are different in terms of their origin and of their potential ability to cause tissue injury. Furthermore, this also suggests that the increased antiphospholipid autoantibody levels observed in this study population do not reflect a general enhancement of Ig production but rather a more specific response directed against anionic phospholipids.

The presence of raised serum levels of antibodies to anionic phospholipids has been found to be associated with a distinct clinical condition, the so-called "antiphospholipid syndrome,"[18] or more specifically, "anticardiolipin syndrome."[19] This includes

Table 2 Levels of Antiphospholipid Antibodies During the Early Follicular Phase Before Treatment

Antigen	Isotype	IVF (n = 35)	Control (n = 36)	Probability[a]
Cardiolipin	IgG	182.3 ± 170.6[b]	69.18 ± 45.0	<0.0001
Cardiolipin	IgM	252.3 ± 126.0	157.0 ± 88.1	0.0008
Phosphatidylcholine	IgG	118.6 ± 50.0	62.0 ± 29.5	<0.0001
Phosphatidylcholine	IgM	136.5 ± 54.0	112.0 ± 59.6	NS[c]
Phosphatidylserine	IgG	213.8 ± 100.0	134.6 ± 77.4	0.0009
Phosphatidylserine	IgM	187.8 ± 121.7	99.3 ± 52.7	0.0003

[a] Calculated using Mann-Whitney test.
[b] Values are means of OD measurements ($\times 10^3$) ± SD.
[c] NS, not significant.

various clinical features such as primary and secondary habitual abortion, intrauterine growth retardation, intrauterine fetal death, thrombosis, and thrombocytopenia, all associated primarily with the presence of the antiphospholipid antibodies in the absence of clinical manifestations of any other autoimmune disease.[20] The mechanism of fetal loss in these patients is unknown, but it has been thought to involve microthrombi formation. The most common pathological findings have been decidual and placental vascular thrombosis. It is suggested that the endothelial cell or the thrombocyte is the target antigen, and the binding of the antibody may induce thrombus formation through mechanisms such as decreased prostacyclin production, inhibition of plasminogen activator, or inhibition of protein C activation.[21,22] The higher incidence of spontaneous abortion reported in patients who conceive after ovulation induction or IVF[9,10] has been attributed mainly to inappropriate endometrial differentiation because of hormonal imbalance that does not permit successful implantation. However, in view of the data presented here, it is tempting to speculate that the increased abortion rate may be related, at least in part, to the high levels of antiphospholipid autoantibodies demonstrated in these patients' sera.

In the present study, levels of all types of sera antiphospholipid antibodies tested (including both IgG and IgM) were uniformly elevated after IVF treatment. Any linkage between individual types of serum antiphospholipid autoantibodies and specific clinical features constituting the antiphospholipid syndrome is yet to be investigated. Originally, all these antibodies were generally identified as lupus anticoagulant because of their ability to prolong in vitro the partial thromboplastin time. Later, after the development of solid-phase assay for antiphospholipid antibodies, it was shown that the anticoagulant effect was mediated by these Igs. It has been claimed that sera exhibiting anticoagulant activity contain antibody specificities against multiple phospholipids; however, this activity is always associated with the presence of antibodies against phosphatidylserine.[23] It is of interest that in the present study, the only naturally occurring antiphospholipid autoantibody that was found to correlate positively with the number of repeated IVF cycles was also antiphosphatidylserine. However, neither the pathogenetic implications nor the clinical significance of these observations is clear.

Based on these preliminary data, it is not yet possible to estimate whether the observed changes in autoantibody levels might have any future clinical influence on infertile patients undergoing IVF treatment. It should be noted that all antiphospholipid autoantibody levels measured in this study fall within the range regarded as normal. The levels demonstrated by IVF patients, although significantly higher than those of their control group, are below the threshold levels considered at present as the cutoff points for diagnosing the respective autoimmune disorders. Further observations concerning the outcome of pregnancies in those patients with significantly raised antiphospholipid antibody levels are of extreme importance and are currently being undertaken in our department. Similarly, a long-term follow-up is required to provide evidence for a possible relationship between the production of antiphospholipid autoantibodies and the development of autoimmune disorders in these patients.

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