Anti-ovary antibodies after attempts at human *in vitro* fertilization induced by follicular puncture rather than hormonal stimulation

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Summary. Anti-ovary antibodies (AOA) have been detected in serum samples of women undergoing *in vitro* fertilization (IVF). High concentrations of these antibodies have been found in women who have had several IVF attempts and they appear to correlate with reduced chances of pregnancy. In this paper, AOA were assayed sequentially in a series of 140 IVF candidates to investigate the respective roles of hormonal stimulation and follicular puncture in inducing the autoimmune response. Serum was obtained 8 days after the beginning of ovarian human menopausal gonadotrophin (hMG) stimulation, then 15 days after follicular puncture. Significantly higher concentrations of IgG ($P < 0.0001$) AOA were observed in the second series of samples than in the first, suggesting that ovarian trauma and not hormonal stimulation is responsible for triggering antibody production. In the whole group, there was a negative correlation between IgM levels after puncture and oocyte numbers ($P < 0.05$). Among 'immune-responder' women, the concentrations of IgA AOA ($P = 0.01$) in the first sample, and of IgG ($P = 0.01$) or IgA AOA ($P < 0.05$) in the second, correlated with fewer oocytes after stimulation. There was no variation in the mean concentrations of AOA in women who achieved pregnancy.

Keywords: human; autoantibodies; ovary; *in vitro* fertilization

Introduction

Numerous aetiologies have been evoked to explain female hypofertility and sterility. Among them immunological causes (McIntyre *et al.*, 1984) have been suspected with increasing frequency, incriminating anti-spermatozoa antibodies (Isojima *et al.*, 1974), systemic anti-cardiolipin autoantibodies (Harris *et al.*, 1984) and, more recently, anti-ovary antibodies. Anti-ovary antibodies had previously been associated with premature menopause, adrenal failure and polyglandular autoimmune disorders (Anhonen *et al.*, 1987; Aiman & Smentek, 1985; Luborsky *et al.*, 1990). A recent report by Moncayo *et al.* (1990), and our own investigations (Gobert *et al.*, 1990; Barbarino-Monnier *et al.*, 1990, 1991) have suggested that such antibodies could be involved both in infertility and in unsuccessful *in vitro* fertilization (IVF) attempts. In an initial series of 110 women attempting IVF, serological anti-ovary antibodies (AOA) were shown to increase with the number of repeated follicular punctures (Barbarino-Monnier *et al.*, 1991). These data indicated that follicular puncture could induce the release of autoantigens not usually encountered by the immune system. Moncayo *et al.* (1989) proposed a different hypothesis suggesting that the production of AOA was induced by hormone-mediated ovarian stimulation and that AOA were directed

against luteinizing hormone (LH) receptors, or human chorionic gonadotrophin (hCG) receptors, or both.

Indirect immunofluorescence, so far the reference method for AOA assessment, shows that AOA are specifically directed to theca interna cells (Sacco & Shivers, 1973; Barbarino-Monnier *et al.*, 1991). This characteristic is consistent with the hypothesis that antigen release occurs after follicular puncture, but evidence is lacking to demonstrate or dismiss a possible leakage of antigenic material in hormone-stimulated ovaries.

In this study we used the enzyme-linked immunosorbent assay (ELISA) method previously reported (Gobert *et al.*, 1990; Barbarino-Monnier *et al.*, 1991) to investigate whether AOA production is triggered by hormonal stimulation of ovaries or by follicular puncture. Our data indicate that the second hypothesis is more likely and further confirm the role of AOA in IVF failures.

Materials and Methods

Patients

Two hundred and eighty serum samples were obtained from 140 women (mean age 31, range 24–40) enrolled in a classical IVF protocol. As a mean the rank of IVF attempts was 2.12, ranging between one and nine. Sixty-six women were punctured for the first time, 33 for the second time. Aetiological causes of infertility were tubal (41%), male factors (27%), idiopathic (19%), endometrial (endometriosis) (6%) or mixed (7%). This partition was similar for women in their first attempt or repeating IVF.

Serum was obtained in each case 8 days after the beginning of ovarian hormonal stimulation using exogenous gonadotrophins (hMG, 75 iu per vial, mean number of vials 31.3 ± 10.2 , range 4–74) and 15 days after follicular puncture. The date of the latter was determined by plasma oestradiol concentrations and ultrasound pelvic examination. Similar numbers of hMG ampoules were necessary in women attempting IVF for the first time (32.5 ± 10) or for those who had previously attempted IVF (30.8 ± 8).

Clinical pregnancy, declared when plasma concentrations of HCG are equal or higher than 1000 iu, or when gestational pouches can be detected by ultrasound pelvic examination, was later observed in 34 patients (24%).

Anti-ovary antibody assay

An ELISA sandwich method, developed in our laboratory and using human ovary soluble crude extract as antigen, was used to assess the amount and isotype of serum AOA. Peroxidase conjugated antibodies to human IgG, IgA and IgM (ICN, Lisle, IL, USA) were used as second-step reagents to identify the amount of each specific isotype. Optical densities (OD) were measured at 492 nm using a Multiskan-Titertek spectrometer (Flow Laboratories, Helsinki, Finland). Results were expressed as the ratio of OD of each serum to the reference OD obtained with a standard pool of control sera. This ratio yields an OD equivalent to the mean + 2 standard deviations of that obtained by the reduced variable method with control samples from normal healthy controls. Values higher than 1 were considered positive.

Statistical analysis

Clinical and biological data were fed to a PC computer, sorted, and analysed using Spearman correlation, paired Student's *t* test or Student's *t* test for independent series, performed with the PCSM software (Deltasoft, Grenoble, France).

Results

AOA on day 8 of hormonal stimulation

Forty per cent of the women had AOA amounts higher than 1 in this first sample studied (Fig. 1). These were IgG in 32 (23%) cases, IgA in 18 (13%) and IgM in 20 (14%). Only 14 women (10%) had significant amounts of more than one isotype, including two with high amounts of all three. Ten of these 14 had already attempted IVF. One of the four women stimulated for the first time had an idiopathic infertility, another one had ovarian dystrophy.

Fig. 1. Partition of anti-ovary antibodies of (a) IgG, (b) IgA and (c) IgM isotype in the serum of 140 women after 8 days of hormonal stimulation (left of each panel) and 15 days after follicular puncture (right of each panel). Data are expressed as OD ratios. The difference between the two groups is significant in paired Student's *t* test for IgG ($P < 0.00001$).

This initial AOA assay also showed higher mean concentrations of IgG ($P < 0.05$) and IgA AOA, and lower concentrations of IgM AOA in the serum of multipunctured women.

No correlation was observed between initial AOA concentration and the numbers of either the vials needed for stimulation, oocytes retrieved after puncture or number of embryos obtained (Table 1).

Table 1. Comparison of the mean numbers of oocytes and embryos obtained in the whole series and various subgroups of 140 women undergoing *in vitro* fertilization

Subgroups	Mean number of oocytes (SD)	Mean number of embryos (SD)
All women	4.8 (3)	2.3 (1.2)
First IVF attempt	4.8 (3)	2.4 (1.1)
Multipunctured	4.7 (2.7)	2.3 (1.3)
IVF success	5.5 (3.8)	2.7 (1)
IVF failure	4.5 (2.3)	2.2 (1.3)

No statistically significant differences were noted.

AOA after follicular puncture

Higher levels of AOA were found in the whole series of serum samples obtained after follicular puncture (Fig. 1). Paired Student's *t* tests indicated that comparisons between the first and second assay were statistically significant for IgG ($P < 0.00001$).

IgG concentrations were significantly higher in serum samples both from women attempting IVF for the first time ($P < 0.009$) or multipunctured ($P = 0.02$) compared with controls, although the difference was smaller in the latter (Fig. 2). The increment in IgG AOA was not significant in

Fig. 2 Increment of anti-ovary antibodies of IgG isotype in the serum of women undergoing *in vitro* fertilization after follicular puncture. Mean concentrations of anti-ovary IgG on day 8 of hormonal stimulation, before follicular puncture (□); mean concentrations of anti-ovary IgG 15 days after follicular puncture (■). A: all 140 women; B: women undergoing *in vitro* fertilization for the first time; C: multipunctured women; D: women whose *in vitro* fertilization attempt led to clinical pregnancy; E: women whose *in vitro* fertilization attempt failed, and women whose follicular puncture yielded less (F) or more (G) than four oocytes. Topping bars indicate standard error values. Between groups comparisons were performed using Student's *t* tests. *Significant difference between the two groups. (*P* values: A < 0.00001; B < 0.009; C < 0.02; E < 0.002; F < 0.003).

the subgroup of women later found pregnant, while it retained statistical significance in the case of IVF failure ($P = 0.002$). Similarly, there was no significant increment in IgG AOA in women for whom more than four oocytes were obtained during follicular puncture, whereas IgG AOA levels increased significantly when less than five oocytes are retrieved ($P = 0.003$). The mean numbers of hMG ampoules given were similar in these two groups of women (respectively 30.7 ± 9 and 32.4 ± 12).

No correlation was observed between AOA levels after puncture and the number of vials that had been necessary for a proper stimulation. However, negative correlations were observed between IgM AOA post-puncture concentrations and both the number of oocytes collected ($P < 0.05$) and of embryos obtained ($P = 0.01$).

A subgroup of 'immune-responder' women

This large series of serum samples screened for the presence of AOA suggests that only some of the women attempting IVF undergo an autoimmune stimulation during this process. This subgroup was named 'immune-responders' and their characteristics were investigated further. Four groups of women were analysed, identified by comparing AOA levels on day 8 of hormonal stimulation and 15 days after puncture and demonstrating increased IgG ($n = 79$), IgA ($n = 75$) or IgM ($n = 74$) AOA, or increment in the three isotypes ($n = 39$). Increased IgA or IgM AOA were associated with significant increases in amounts of the other isotypes. In these subgroups, strong negative correlations were observed between high AOA levels and low numbers of oocytes.

Interestingly, even in these subgroups of immune responders, the increase observed was not statistically significant when women who later became pregnant are considered.

Discussion

Four issues were addressed in this study: the role of hormonal therapy as inducer of AOA, the relationship between follicular puncture and AOA production, the bearing of AOA on the subsequent outcome of IVF, and the presence of AOA in IVF candidates as a possible cause of infertility.

A significant difference in IgG levels was observed in this series when comparing AOA concentrations before and after follicular puncture. This suggests that hMG-induced ovarian stimulation is not alone responsible for the induction of anti-ovarian immunity, and further indicates that follicular puncture can induce an immune response. Primary responses in women undergoing IVF for the first time, or secondary responses in multipunctured women would have resulted in significantly high concentrations after 8 days of stimulation. Concentrations measured in the second sample tested (15–21 days later) would therefore have been similar or decreased. Interestingly, more than half the women tested in this series could be considered immune responders, and had low AOA concentrations after 8 days of hormonal stimulation.

The hypothesis that hormonal stimulation is responsible for the induction of AOA had been proposed as an explanation for the raised concentrations of anti-bovine ovary antibodies reported by Moncayo *et al.* (1989). However, the rank of IVF attempt and infertility aetiology were unknown in this group of positive women. In the series reported here, significantly higher concentrations of AOA were however observed in the first sample tested obtained from women repeating the IVF procedure. These data further confirm those previously reported by our group (Gobert *et al.*, 1990; Barbarino-Monnier *et al.*, 1990, 1991), suggesting that follicular puncture may be involved in triggering anti-ovarian immunity.

Regarding the outcome of IVF, we found a relationship between AOA and both the number of oocytes and the development of clinical pregnancy. In both instances, high numbers of oocytes and IVF success were associated with stable and lower levels of AOA. Although indirect evidence, these data suggest that AOA may interfere during the maturation of follicles or oocytes. Interestingly, no correlation was found between high concentrations of IgG or IgA AOA and the number of fertilized eggs. This could be explained by the fact that the oocytes are fertilized *in vitro* with the spermatozoa of the husband or donor, thus without any contact with the female immune system. The mature oocytes collected thus appear to be fully competent. However, upon reimplantation of the embryos, their development depends on proper follicular functions, again possibly impaired by AOA. This hypothesis is consistent with the high concentrations of AOA encountered in IVF failure and, conversely, with the low concentrations of AOA associated with the development of clinical pregnancy.

Finally, AOA were observed in 21% of the women tested during their first attempt at IVF. These data are consistent with an autoimmune aetiology for their infertility. It could be relevant that infertility had been tagged 'idiopathic' in six of these women. How ovarian auto-antigens could trigger the immune system in these cases is still unknown.

In conclusion, our data suggest that AOA are induced in the course of IVF by follicular puncture and not by hormonal therapy. They may be a consequence of a surgical release of auto-antigens and appear to interfere with conception and embryo development. These autoantibodies can be found in women who are not engaged in an IVF protocol and may thus be associated with certain idiopathic sterilities. The assessment of these autoantibodies could be a useful method for monitoring IVF and could be used to investigate immunological infertilities.

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Received 5 August 1991