ORIGINAL ARTICLE

Autoantibody profile and other immunological parameters in recurrent spontaneous abortion patients

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ABSTRACT

Background: An autoimmune cause and related immunological alterations resulting in recurrent spontaneous abortion (RSA) have been suggested in patients with unknown etiology. **Materials and Methods:** This study evaluated the autoantibody profile and other immunological parameters among RSA patients and normal pregnant women from Mumbai western India. Fifty RSA patients with unknown cause and greater than three consecutive abortions along with 50 normal pregnant women were studied for various auto antibodies such as ANA, anti-dsDNA, ANCA, AECA, 2 micro globulin, anti-HLA antibodies and ACLA using immunofluorescence microlymphocytotoxicity and ELISA. Immunological parameters such as HLA class I monoclonal antibody expression, CD3 (T cell), CD19 (B cell), and CD56 (NK cell) were estimated by flow cytometry. **Results:** The results revealed 34% positivity of all auto antibodies tested among patients. ANA(12%), ANCA (20%), AECA (24%), ACLA (8%), anti-dsDNA(0%), β2 microglobulin (14%), and anti-HLA antibodies(10%) among RSA patients were identified. An increased expression of HLA class I specific monoclonal antibody (10%) with HLA A3 (16%) specificity were found to correlate with shared HLA alleles among the RSA couples. Among normal pregnant (control) group ANA (2%), ANCA (2%), AECA (3%), ACLA (4%) and increased expression of CD56 with reduced HLA class I monoclonal were observed. **Conclusion:** Our findings suggest a possible role of various autoantibodies along with the related immunological parameters underlying RSA.

Key words: Autoantibody, Human leukocyte antigen, India, Recurrent spontanous abortion

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INTRODUCTION

Normal mammalian pregnancy is confronted with a great number of self (autoimmune) and foreign (alloimmune) antigens that modulate the immune system of the mother when maternal immune response is affected, recurrent pregnancy loss can result. Recurrent spontaneous abortions (RSA) resulting in three on more consecutive pregnancy losses prior to the 20th week of gestation are seen in about 5% in Indian mothers.[1]

The etiology of RSA is often unclear and may be multi-factorial. The reasonable etiologic causes include genetic, anatomical, endocrine, placental anomalies, hormonal problems, infections, etc. Recently, autoimmune factors have been recognized as a key factoring recurrent pregnancy loss, even in women with no clinically diagnosed classical autoimmune disease. The concept that these losses may result from immunorejection similar to that seen in allograft transplantation. An interest in an autoimmune cause of RSA has increased greatly with the association of various autoantibodies and related immunological parameters with recurrent spontaneous abortion. Among most of these patients it has been suggested that autoantibodies may be responsible for immunological alterations leading to RSA.[2] Therefore, the autoantibody profile and other immunological parameters among RSA patients and normal pregnant women from Mumbai western India were studied.

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MATERIALS AND METHODS

This prospective study obtained KEM Hospitals Ethical committee approval included 50 RSA patients who were clinically examined by gynecologists, of KEM hospital. The inclusion criteria for unexplained RSA were defined by three or more consecutive pregnancy losses before 20 weeks gestation. The patients age ranged between 20 and 37 years with mean + SD of 26.5 + 2.5 years. A

group of 50 normal pregnant women having no previous history of pregnancy loss was also included as a control group. Exclusion criteria were HIV positivity, cytogenetic abnormalities as well as positivity for anti-sperm and anti-ovum antibodies.

Autoantibodies such as anti-nuclear antibodies (ANA) were qualitatively and quantitatively tested by IIF using HEp-2 cells and a cut off for positivity was 1: 20 dilution for ANA testing.[3] Anti-neutrophil cytoplasmic antibodies (ANCA) were also tested with a cut off of 1: 10 dilution of the test sample by the IIF method using human neutrophils (PMN) with ethanol and formalin-fixed preparations and the slides were probed with FITC tagged polyvalent anti-human globulin using a fluorescent microscope.[4] All the test samples giving fluorescence were further examined on the confocal laser scanning microscope (LSM 510, Karl Zeiss, Germany).[5] Autoantibodies to double-stranded DNA (anti-dsDNA), anti-Cardiolipin antibodies (ACA) were detected by ELISA using commercial kits. (Genesis, UK)[6] Anti-HLA antibodies were detected by the microcytotoxicity technique[7] and anti-2 microglobulin antibodies were detected by flow cytometry.[8]

In order to detect anti-endothelial cell antibodies (AECA), endothelial cells (ECs) were isolated from sterile human umbilical cords (HUVEC) by collagenase (0.2% v/v) digestion of interior walls of umbilical veins by introducing through the umbilical catheter.[9] The cells were grown in tissue culture medium IMDM (Sigma, USA), supplemented with 10% FCS (Gibco, Scotland) supplemented with 2 ml glutamine, 100 U penicillin/ml and 100 g of streptomycin/ml. The culture plates were incubated at 37 °C under 5% CO_2 atmosphere. The cells were fed at 2 day intervals and morphology was confirmed by phase contrast light microscopy showing the typical cobblestone monolayer appearance of endothelial cells. The confluent cells after second passage were used after giving two washes with PBS (0.01 M, p. 7.2) and adjusting the cell count to 1×10^6/ml. Homogenous population of actively growing ECs (>90% von Willibrand factor antigen positive) on second passage was obtained, which was detected by using anti-vWF monoclonal antibody (Dako, Denmark) by indirect immunofluorescence test (IIF).[22]

Immunophenotypic profiles defined using peripheral blood mononuclear cells (PBMCs) for cellular autoimmune response, major lymphocyte subsets of T cells (CD3+), B cells (CD19+) and NK cells (CD3-/CD16+56) were detected by flow cytometry. Immunophenotypic profiles were assessed by multiparameter analysis based on triple color immunophenotypic study of surface antigen using combination of CD56-phycoerythrine (PE), CD19-fluoresceine isothiocynate (FITC) and CD3-PerCP-conjugated monoclonal antibodies. Mononuclear cells were separated by the density gradient method and cell count was adjusted to 2×10^6 in 100 µl where 100 µl of monoclonal antibodies added to each tube along with respective isotype controls. After mixing, tubes were incubated at 4 °C for 20 min in dark. After giving wash with PBS (0.01M, p. 7.2), cells were resuspended in 500 µl PBS to acquire and further analysis on a flowcytometer.

RESULTS

The overall positivity of autoantibodies was found to be 34% in RSA patients. ANA positivity of 12%, ANCA positivity 20%, AECA positivity of 24%, and ACLA positivity of 8% was found. Anti-dsDNA autoantibodies were absent in these patients. Autoantibodies to 2 microglobulin were found in 14% patients. Anti-HLA antibodies were found in 10% patients. An increased expression HLA class I monoclonal antibodies (16%) specific for HLA A3 allele (odds ratio: 10.75; c^2: 5.44; *P* value: 0.01; confidence interval: 1.30-88.51) which correlated with the shared HLA alleles among the couple. These autoantibodies were also detected in normal pregnant (control) group where ANA positivity was found in 2%, ANCA positivity in 2%, AECA in 3% and ACLA positivity in 4% patients. Increased HLA expression was not detected in any of the control group patients [Table 1]. Immunophenotypic studies on lymphocyte subsets revealed that the reduced expression of CD3, CD19, and CD56 was found in 10%, 18%, and 12% patients respectively where as an increased expression of CD3, CD19, and CD56 were found in 10%, 12%, and 16% as compared to controls [Table 2].

DISCUSSION

Immune mechanisms for recurrent spontaneous abortions have been explored without coherent results. Recently, an association between RSA and the presence of a specific autoantibody or patterns of autoantibodies has been established. Although majority of RSA remains unexplained, it is found to be associated with autoantibodies such as anti-phospholipid antibodies (APA), anti-nuclear

Table 1: Auto-antibody profile among RSA patients from Mumbai, India

Autoantibodies	Patients *N*=50	Controls *N*=50		Ki2	95% CI	*P* value
	PF(%)	PF(%)	OR			
ANA	10.00	4.00	2.66	0.61	0.49-14.45	0.22
ANCA	20.00	4.00	6.00	4.64	1.24-28.99	0.02
AECA	26.00	2.00	17.22	10.05	2.15-137.64	0.0008**
ACLA	20.00	2.00	12.25	6.34	1.50-99.85	0.01
Anti-b2 microglobulin	14.00	2.00	6.68	2.45	0.77-57.72	0.06
Anti-HLA antibodies	10.00	10.00	1.00	0.00	0.27-2.69	1.00

** Significant *P* value, Ki2 = Chi-square with Yates correction, PF(%) Phenotype frequency percent, OR = Odds ratio, 95% CI = 95% confidence interval, RSA = Recurrent spontaneous abortion

Table 2: Other immunological parameters in RSA patients

Immunological parameters	Number positives	% positivity	Cut off values (average)	Normal range
Increased MHC expression				
Class I	8	16	0.9	0.3-2.9
2 microglobulin	7	14	1.02	0.1-2.6
Increased CD expression				
T cells (CD_3)	5	10	52.2	23-81.5
B cells (CD19)	6	12	8.5	5-12
NK cells (CD56)	24	48	22.5	1.2-44

RSA = Recurrent spontaneous abortion

antibodies (ANA), anti-cardiolipin antibodies (ACA), anti-neutorphil cytoplasmic antibodies (ANCA), anti-thyroid antibodies (ATA), and anti-endothelial cell antibodies (AECA). These autoantibodies are found to be associated with immunological failure of pregnancy leading to abortion.[10] Kovacs *et al*. in 1999 have also reported that immunologic mechanisms detected in classical autoimmune disease like systemic lupus erythematosus (SLE) may also operate in a subgroup of habitual abortors with suspected immunological cause, where ANA, ANCA, and APA were found to be present in RSA patients.[9] Xu *et al*., 1990, reported significance of low level ANA titers to be associated with a high risk pregnancy loss.[11]

Unexplained RSAs has many etiologies, many of them have immune dysfunctions which include the presence of cytotoxic antibodies, absence of maternal blocking antibodies, sharing of HLA antigens, and disturbances of killer cell immunoglobulin-like receptor dysfunction. The roles of immunological and genetic characters have been implicated in RSA. The HLA alleles A*030101, B*5701, Cw*120201, DRB1*030101, and DRB1*150101 as well as their associated ancestral haplotype may play a significant role in development of RSA in India. Earlier, we had reported HLA A3 association in RhD immunized women from western India.[12] HLA B17 has been reported to be associated in Italian RSA patients.[13] HLA haplotype A1-B17 has been reported to be involved in antineutrophil cytoplasmic antibodies (ANCA) production and A*0101-B*5801 haplotype is significantly associated with autoimmune disease from western Indian population.[14] A significant reduced frequency of HLA B35 has been described in Japanese RSA patients.[15] Among Roman RSA couples, significant differences in HLA A2, B18, and B40 have been reported.[16] Increased frequencies of Cw5, Cw6, and DR2 in Italian RSA patients have been reported.[17] Further among the UK RSA patient population linkage disequilibrium between A2 and B12 has been reported.[18] Reports on antigen sharing in couples with recurrent miscarriage led to several studies assessing the role of HLA antigens and their influence on the outcome of pregnancy.[19] In fact, couples sharing at HLA A, HLA B, HLA C, HLA DR, and HLA DQ loci have been reported to be positively associated with the risk of RSA.[20] Since 1977, increased HLA sharing among spouses has been associated with RSAs; later more, specific HLA DR and/or DQ antigens were suggested. HLA sharing has also been reported in couples that fail to achieve pregnancy with multiple cycles of assisted reproductive techniques. The study investigating on HLA allele sharing between RSA partners, the maternal killer immunoglobulin-like receptor (KIR) repertoire, and repeated implantation failure after *in vitro* fertilization (IVF)/embryo transfer suggested that increased HLA sharing per se or a limited maternal KIR repertoire predisposes to RSA or IVF failure. A slightly higher percentage of DQA1*0505 sharing in the RSAauto and the IVF group. The ratio of inhibitory to activating KIR (actKIR) was slightly lower in RSAallo and IVF women (1.9 vs. 2.6 in controls), while in a high percentage of these women, the standard receptors of the KIR A haplotype were combined with actKIR/s of the haplotype B (66.6% and 45.4% vs. 20% and 15.3% in RSA auto and control groups) suggesting a possible involvement of actKIRs in embryo implantation and the maintenance of pregnancy.[21]

Our study showed a significant increase in autoantibody formation in RSA patients and the prevalence of autoantibodies in RSA patients from India. ANCA and AECA are associated immunopathologically with vascular damage. ANA are found to be associated as a result of secondary autoimmune phenomenon in RSA patients and ACLA are found to be associated with increased tendency to thrombus formation. The upregulation of HLA class I-specific monoclonal antibody in RSA patients suggest HLA allele restricted immune response. Hence, a better understanding of these underlying mechanisms resulting in RSA and the effect of these autoantibodies on growing fetus after they cross the placenta needs to be explored in order to create effective immunotherapies.

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