The occurrence of immunological disturbances in patients with recurrent miscarriage (RM) of unknown etiology

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Abstract

OBJECTIVE: The 155 patients suffering from primary RM who took part in this study were qualified after excluding known causes of abortions.
METHODS: The presence of the following immunological factors was examined in serum samples: autoantibodies such as anti-cardiolipin (ACA) IgG and IgM, lupus-anticoagulant (LA), anti-thyroid (ATA): anti-thyroglobulin (anti-TG) and anti-thyroid peroxydase (anti-TPO), anti-nuclear (ANA), anti-placental (APA) antibodies and alloimmunological disturbances following HLA-class I antibody test (LCT-lymphocytotoxic test), lack of blocking proliferative activity in mixed lymphocyte reaction test (MLR), anti-sperm (ASA) antibodies, levels of extracellular interferon γ (IFN γ) and tumour necrosis α (TNFα) produced by peripheral blood lymphocytes.
RESULTS: Immunological disturbances were found in 69.1% of the patients suffering from primary RM of unknown etiology. The number of RM correlated with the frequency of immunological disturbance. Among the samples from patients who underwent ≥5 RM, positive levels of TNFα were the most frequently observed risk factor (up to 27%) (p=0.05). Although the incidence of immunological factors was comparable between patients with early and late pregnancy losses, elevated ATA levels were frequently seen among late miscarriage (95% CI=36.0, $p \leq 0.01$).
CONCLUSION: The heterogeneity of immunological risk factors shown in this study indicates the usefulness of detecting alloimmune factors as well as autoantibodies in patients with RM of unknown etiology. This may be helpful to analyse the therapeutical effectivity of various treatment in a better characterized group of patients and to explain unsuccessful results of treatment in patients with RM of unknown etiology.

ORIGINAL ARTICLE

Abbreviations:

ACA antibodies - anti-cardiolipin antibodies
ANA antibodies - anti-nuclear antibodies
anti-TPO antibodies - anti-peroxydase antibodies
anti-Tg antibodies - anti-thyroglobulin antibodies
APA antibodies - anti-placental antibodies
ASA antibodies - anti-sperm antibodies
ATA antibodies - anti-thyroid antibodies
CI - confidence interval
cpm - count per minute
FSH - follicule stimulating hormone
HLA - human leukocyte antigen
HSG - hysterosalpingography
IFNγ - interferon gamma
Ig - immunoglobulin
LA - lupus anticoagulant
LCT - lymphocytotoxic test
LH - luteinizing hormone
MLR - mixed lymphocyte reaction
OR - odds ratio
p - Pearson factor
pg - picogramma
PRL - prolactine
RM - recurrent miscarriage
TNFα - tumour necrosis alfa
TORCH - Toxoplasmosis, other infectious microorganisms, Rubella, Cytomegaly, Herpes simplex
TSH - thyroid stimulating hormone
U - unit international

INTRODUCTION

In previous studies, approximately 65% of total RM (recurrent miscarriage) and 80% of unexplained RM were found to be related to disruptions in the immunologic balance between mother and foetus (Coulam, 1991; Sierra and Stephenson, 2006; Christiansen *et al.* 2008). One of the most discussed causes for the failure to conceive has become the imbalance of Th1/Th2 cytokine activity (Wegman *et al.* 1993), as well as both auto and alloimmunological disturbances (Toth *et al.* 2010). Genetic polymorphisms associated to HLA class I and class II alleles and genetic polymorphisms of NK cell interactions or cytokine production can be found with increased prevalence in RM patients (Christiansen OB. *Et al.* 2008). The known possible alloimmune cause for RM is based on increased sharing of human leukocyte antigen (HLA) between both partners which prevents the maternal production of blocking antibodies protecting the foetus (Malinowski *et al.* 1997; Kotlan *et al.* 2006). Most of autoimmune disturbances have a predilection for women in their reproductive years, supporting concept that pre-clinical autoimmune disease can cause reproductive failure.

The aim of this study was to investigate immunological disturbances in Polish women suffering from RM of unknown etiology and to define which differences of the immune factors could be advantageous for higher efficacy in the treatment.

MATERIALS AND METHODS

Study population

The test group consisted of 155 women with a history of 3 or more consecutive primary miscarriage before the 22nd gestational week, registered at the Department of Operative and Endoscopic Gynecology, Polish Mother's Memorial Hospital – Research Institute, Lodz from May 2009 to May 2012. The tested group consisted of nonpregnant women with RM, in whom we could find no reason for abortion either in case histories or in clinical and laboratory investigations. Patients taking part in the study were the primary aborters – previous miscarriage occurred before 20 weeks of gestation with the same partner. Research was carried into all these women to exclude the known causes of RM: a previous history of miscarriage, family history, clinical examination and BMI, genetic abnormalities (both the women and their partners also had karyotype analysis), congenital and acquired uterine anomalies, endocrinopathies (luteal phase insufficiency, polycystic ovary syndrome, insulin resistance and hyperandrogenism, diabetes mellitus, thyroid and prolactin disorders) in the result of hormonal researches, *TORCH* and cervical infections, enviromental factors (toxins, alcohol, cigarettes, caffeine), heritable thrombophilias connected to RM and no evidence for autoimmune connective tissue disease. Chromosomal abnormality of miscarriage tissue was excluded using cytogenetic analysis for chromosomes 13, 16, 18, 21, X and Y in patients suffering subsequent abortions starting at the second pregnancy loss.

The patients' ages ranged from 22 to 45 years (mean age 31) and the number of abortions ranged from 3 to 7. They had normal and standard medical and gynaecological examinations. All the patients who took part in our study didn't underwent previous treatment. The partners of these women had normal spermiograms. Patients were tested immunologically after excluding known reasons of RM (Table 1). All patients enrolled in this study have negative values of lymphocytotoxic tests (LCT) and were lacking of blocking proliferative activity in mixed lymphocyte reaction test (MLR) (Malinowski *et al.* 1997; Malinowski, 2001). Tests were performed prior to possible pregnancy. The presence of the following immunological factors was examined in serum samples: autoantibodies such as anti-cardiolipin (ACA) IgG and IgM, lupus-anticoagulant (LA), antithyroid (ATA) – anti-thyroglobulin (anti-TG), antithyroid peroxydase (anti-TPO), anti-placental (APA) and anti-nuclear (ANA) antibodies. Anti-sperm (ASA) antibodies, levels of interferon γ (IFN γ) and tumour necrosis α (TNFα) produced by peripheral blood lymphocytes were also examined.

As controls, 50 healthy and fertile women who had delivered at least 1 year ago were recruited. LCT test and MLR test were not performed in the control group. The Institutional Review Board for Clinical Investiga-

tion approved the study and informed consent was obtained from all participants.

Laboratory

Ten millitres of heparinized peripheral blood was drawn from RM women and the control group using a Vacutainer sampling system (Beckton-Dickinson, Germany). Peripheral blood samples were drawn into tubes in the absence of an anticoagulant. Samples were allowed to clot and were centrifuged thereafter. The serum samples were aliquoted and kept at –20 °C until used.

LCT (lymphocytotoxic test): The measurement of serum anti-HLA class I IgG (ELISA lymphocytotoxic test) Quick Screen Solid Phase HLA Antibody Screening Kit QS12G (GTI,USA) was used. Lymphocytotoxic test value of <0.30 U/ml was considered to be negative.

Lymphocyte proliferative response in mixed lymphocyte reaction (MLR): Ten millitres of heparinized peripheral blood was drawn from RM women and their partners. Blood was collected under sterile conditions and peripheral blood cells were isolated from heparinized whole blood by density gradient centrifugation on Ficoll-Hypaque gradient. Responding lymphocytes of women with RM were stimulated with ^{60}Co-irradiated (3000 rad) partner'rs (husband's) lymphocytes. The cells were cultured for 120 h at 37^0 C and 5% CO2 atmosphere and their proliferation was evaluated. The results describing the influence of serum of the examined women on the proliferative response in MLR in comparison with the proliferation in the allogenic serum were shown as the percentage of MLR blocking activity (%MLR blocking) as a formula:

% MLR blocking = (1 – BI) × 100%, with BI = (cpm in autologous serum – control cpm) / cpm in allogenic serum – control cpm).

The value above 22% of MLR blocking was recognized as a presence of the factors blocking significantly the proliferative activity of the blocking antibodies (MLR-BAbs) (Malinowski *et al.* 1997).

Anti-cardiolipin Antibodies (ACA) were detected using a Diagnostic Division Varelisa IgG and IgM Antibodies Immunoassay ELISA (Pharmacia Deutschland GmbH). The results were reported as positive when the following cutoff values were met: >12 GPL-U/ml for IgG and 6 MPL-U/ml for IgM.

Samples that initially tested positive were retested after 12 weeks, according to the Sydney criteria (Miyakis *et al.* 2006).

Lupus anticoagulant (LA): The concentration of serum lupus anticoagulant (LA) was estimated with LAC screen and LAC confirmation tests (Instrumentation Laboratory, Lexington, USA). Values were given as normalized LAC coefficient : LA(–) <1.2, LA(+) 1.2–1.5, LA(++) 1.5–2.0, LA(+++) >2.0. Results greater than 1.2 were reported as positive.

Anti-thyroid antibodies (ATA): anti-thyroglobulin (anti-Tg) and anti-thyroidperoxidase (anti-TPO) antibodies were detected using a Diagnostic Division Varelisa IgG and IgM Antibodies Immunoassay Elisa (Pharmacia Deutschland GmbH). Titers over 60 IU/ml for anti-Tg and for anti-TPO were considered to be positive.

Anti-placental antibodies (APA) were studied using indirect immunofluorescence together with placenta cells as a targets (Euroimmune). Sera that exhibited fluorescence at a dilution of 1:10 were considered positive.

Anti-nuclear antibodies (ANA) were studied using indirect immunofluorescence together with laryngeal cancer HEP-2 cells cultures as targets (Euroimmune). Sera that exhibited fluorescence at a dilution of 1:80 were considered positive.

Interferon γ and tumour necrosis α. Lymphocytes for cytokine secretion were isolated from peripheral heparinized whole blood by Lymphoprep gradient

Tab. 1. Exclusion criteria for RM patients.

Parental karyotype analysis	chromosomal aberrations
HSG and/or hysteroscopy, two- and three-dimensional ultrasound	congenital uterine anomalies: incomplete müllerian fusion , septum resorption, cervical incompetence, acquired anatomical abnormalities of the uterus: synechiae, leiomyomas, cervical incompetence
endocrinopathies	female hormonal researches (LH, FSH, TSH,fT4, fT3, PRL, estradiol, testosterone, androstendion) 6–7 day of cycle, progesterone 23–25 day of cycle
TORCH and cervical infections	TORCH (Toxoplasmosis, other infectious microorganisms, Rubella, Cytomegaly, Herpes simplex), Listeria monocytogenes, Bacterial Vaginosis active infection, cervical infections (Chlamydia trachomatis, Mycoplasma hominis, Ureaplasma urealyticum)
endometrial biopsy	acute or chronic endometritis
heritable thrombophilias	activated protein C resistance (APCR) associated with mutations in factor V Leiden, deficiency in protein C and S, mutations in prothrombin 20210 A, mutations in antithrombin III, hyperhomocysteinemia
OGTT 75 test	glucose intolerance, insulin resistance or diabetes mellitus
Chromosome assessment of miscarriage tissue	trisomy, monosomy or polyploidy, molar pregnancy

separation (Nycomed Pharma, Poland). Lymphocyte suspension of 1×10^6 cells were prepared and cultured in a medium enriched with 2 mM L-glutamine, 10% foetal calf serum and antibiotics (100 U/ml Penicillin, 10 µg/ml Streptomycin; Sigma, St. Louis, USA). Cultures were stimulated with phytohemagglutinin in mitogenic dose of 5 mg/ml (Sigma, St.Louis, USA) and incubated for 24 hours at 37 °C. After centrifugation the supernatant was collected for the cytokine assay. IFNγ and TNFα production was estimated using BD™ Cytometric Bead Array Human Th1/Th2 Cytokine kit (Beckton-Dickinson and Co., BD Biosciences, San Jose, USA). Tests were performed on FacsCanto cytometer (Beckton-Dickinson and Co., BD Biosciences, San Jose, USA) using FCAP Array Software v.101. Concentration of cytokines was given in pg/ml. The reference values considered as positive for IFN γ was 290 pg/ml and for TNF α 323 pg/ml.

Anti-sperm antibodies (ASA) were detected using a Diagnostic Division Varelisa Antibodies Immunoassay ELISA (Pharmacia Deutschland GmbH). The results were considered positive for titres over 20 U/ml.

Statistical analysis

Pearson's chi-squared and Student's *t* test were employed for testing qualitative and continous variables. Statistical significance was set as $p\leq0.05$. Computation was performed using WinBUGS software (Spiegelhalter *et al.* 2004). The computation for cluster analysis was performed using R software (The R Foundation for Statistical Computing). The R version used was 2.14.2, 2011.

RESULTS

In this study, 155 patients with primary RM and 50 control patients were analysed. The median age of the RM patients was 31 years, the median age of control patients was 29 years. The total patient results are presented in Table 2. The results show the percentage of patients suffering from RM that tested positive for autoantibodies, ASA, cytokines IFNγ and TNFα produced by peripheral blood lymphocytes, apart from negative lymphocytotoxic test (LCT) and lack of blocking proliferative activity in mixed lymphocyte reaction test (MLR).

Tab. 2. Results for RM patients compared with controls.

Immunological factors	RM (n=155)	control (n=50)	*p*-value
ACA	25 (16.1%)	0	<0.01*
LA	21 (13.5%)	0	<0.01*
ATA	34 (21.9%)	8 (16.0%)	0.37
ANA	29 (18.7%)	5 (10.0%)	0.15
APA	23 (14.8%)	2 (4.0%)	0.04*
ASA	35 (22.6%)	4 (8.0%)	0.02*
IFNγ	53 (34.2%)	0	<0.01*
TNFα	20 (12.9%)	2 (4.0%)	0.07

RM – recurrent miscarriage, ACA – anti-cardiolipin antibodies, LA – lupus anticoagulant, ATA – anti-thyroid antibodies, ANA´– anti-nuclear antibodies, APA – anti-placental antibodies, ASA – anti-sperm antibodies, IFN γ – interferon gamma, TNF α – tumour necrosis alfa, n – number of subjects, *p* – Pearson's chi-square test, *statistically significant.

Anti-cardiolipin antibodies (IgM and IgG) were found in 16.1% of RM patients, the frequency of lupus anticoagulant detected in serum samples was 13.5%. The anti-thyroid antibodies (anti-Tg and anti-TPO) were found in 21.9% of serum samples, compared to 16.0% of controls. All women found to be positive for TPO-Ab or/and TG-Ab were euthyroid with normal TSH level (0.27–4.2 mIU/ml). In euthyroid women with thyroid peroxidase antibodies was applied levothyroxine 50mcg oral once daily started pre-conceptually and continued to the end of next pregnancy.

Anti-placental antibodies were detected in 14.8% of RM patients but only in 4.0% of sera of control patients. Tested serum samples were positive for anti-nuclear antibodies in 18.7% of RM patients and in 10% of control patients. The frequency of circulating anti-sperm antibodies in RM patients' and control patients' sera was 22.8% and 8.0%, respectively. Positive values of IFNγ were found in sera of 34.2% RM patients, positive values of TNFα were present in serum samples from 12.9% of RM patients and in 4% of control patients.

Of the 155 total serum samples examined and controlled for a lack of blocking proliferative activity in MLR test, 69.1% scored positive for immunological disruptions. The number of RM correlated with the frequency of immunological disturbances: immunological disruptions were found in 55.9% of patients with 3 miscarriage, in 62.79% of patients with 4 and in 66.6% of patients with ≥5 miscarriage as presented in Table 3. Among the samples from patients who underwent ≥5 RM, positive levels of TNFα were the most frequently observed risk factor (up to 27%) (p=0.05).

RM were classified as early pregnancy loss with ultrasound definition of intrauterine pregnancy with reproducible evidence of lost fetal heart activity and/or failure of increased crown-rump length over one week or persisting presence of empty sac at less than 12 weeks' gestation and late pregnancy loss after 12 weeks gestational age where fetal measurement was followed by loss of fetal heart activity. The probability of immunological disturbances was correlated to the gestational age which is shown in Table 4.

Although the incidence of immunological factors was comparable between patients with early and late RM, elevated ATA levels were seen frequently among patients suffering from late pregnancy loss (95% CI=36.0, $p\leq0.01$). Early miscarriage were more frequently observed between 7 and 8 weeks of gestation what is shown in Figure 1.

Considering the heterogeneity of immunological disruptions in RM patients the similarity between the

Tab. 3. Distribution of immunological disturbances among cases according to the number of miscarriage.

Immunological factors	3 miscarriage (n=120)	4 miscarriage (n=43)	≥5 miscarriage (n=21)	*p*-value
	probability % (95% CI)			
ACA	10.2 (9.4, 12.2)	10.3 (9.2, 14.3)	10.3 (9.4, 12.3)	0.43
LA	15.1 (12.9, 18.2)	13.0 (10.3, 15.1)	11.2 (10.1, 13.6)	0.24
ATA	23.4 (15.6, 32.4)	20.1 (12.3, 29.0)	18.0 (6.1, 35.0)	0.26
ANA	18.9 (11.1, 28.3)	18.7 (10.4, 28.6)	19.6 (5.8, 40.3)	0.49
APA	12.3 (6.8, 19.2)	17.2 (10.8, 24.9)	24.4 (10.3, 42.7)	0.09
ASA	20.6 (13.1, 29.5)	24.6 (14.8, 35.5)	29.8 (10.7, 53.8)	0.24
IFNγ	31.0 (19.4, 44.0)	36.4 (24.8, 48.8)	42.6 (20.7, 66.5)	0.22
TNFα	8.6 (3.0, 17.2)	14.9 (7.5, 24.5)	26.4 (8.2, 50.6)	0.05

ACA – anti-cardiolipin antibodies, LA – lupus anticoagulant, ATA – anti-thyroid antibodies, ANA – anti-nuclear antibodies, APA – anti-placental antibodies, ASA – anti-sperm antibodies, IFN γ – interferon gamma, TNF α – tumour necrosis alfa, n – number of subjects *p* – Pearson chi-square test, CI – confidence interval

Tab. 4. The occurrence of immunological factors in early and late pregnancy loss.

Immunological factor	Early RM	Late RM	*p*-value
	probability % (95% CI)		
ACA	11.4 (10.2, 14.3)	9.3 (7.4, 10.4)	0.15
LA	13.2 (12.8, 15.1)	15.1 (13.8, 18.2)	0.38
ATA	17.0 (10.5, 24.9)	36.0 (21.3, 52.3)	≤0.01 *
ANA	18.6 (11.2, 27.5)	19.2 (6.9, 36.1)	0.49
APA	16.6 (10.4, 24.0)	10.2 (2.9, 21.1)	0.14
ASA	22.3 (14.7, 30.9)	21.3 (8.3, 38.2)	0.43
IFNγ	36.9 (25.1, 49.8)	27.5 (12.9, 45.)	0.18
TNFα	14.0 (6.3, 23.9)	10.3 (2.2, 23.6)	0.28

early RM – recurrent miscarriage between 5–12 weeks of gestation, late RM – recurrent miscarriage between 13–20 weeks of gestation, ACA – anti-cardiolipin antibodies, LA – lupus anticoagulant, ATA – anti-thyroid antibodies, ANA – anti-nuclear antibodies, APA – anti-placental antibodies, ASA – anti-sperm antibodies, IFN γ – interferon gamma, TNF α – tumour necrosis alfa, CI – confidence interval, *p* – Pearson chi-square test, *statistically significant.

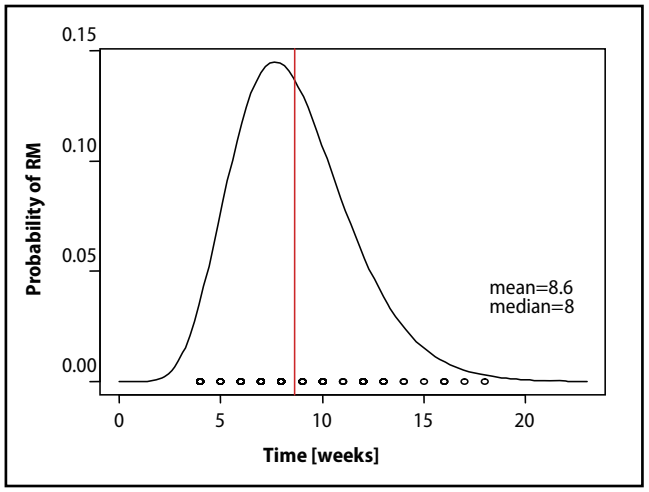

Fig. 1. The probability of time of abortion (weeks) in RM patients.

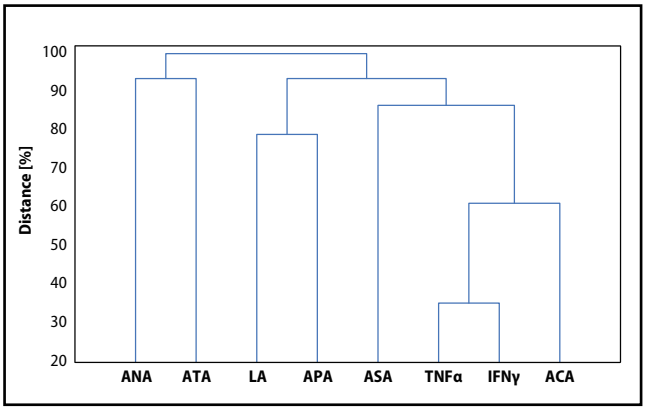

Fig. 2. Cluster analysis for immunological disturbances in RM patients (dendrogram). ANA-anti-nuclear antibodies, ACA- anti-cardiolipin antibodies, ATA – anti-thyroid antibodies, APA – anti-placental antibodies, LA – lupus anticoagulant, ASA – anti-sperm antibodies, IFN γ – interferon gamma, TNF α – tumor necrosis alfa.

analysed immunological disturbances was studied. The analysis was based on cluster analysis (Euclidean distances). The results are shown in the dendrogram in Figure 2. A strong similarity was observed between positive levels of extracellular cytokines TNFα and IFNγ produced by peripheral blood lymphocytes. Among the autoimmune disorders the simultaneous presence of anti-nuclear antibodies (ANA) and anti-thyroid antibodies (ATA) was noted, similar to anti-placental antibodies (APA) and lupus anticoagulant (LA).

DISCUSSION

In this study, we demonstrate the distribution of different immunological factors in women suffering from RM of unknown etiology, who had negative HLA-class I antibody tests (LCT) and lack of blocking proliferative activity in mixed lymphocyte reaction test (MLR).

The local materno-foetal inflammatory response affects the regulatory functions of NK cells and Th1/Th2 cytokines at the implantation site leading to the eventual loss of maternal-foetal tolerance (Plaks *et al.* 2008; Hanzlikova *et al.* 2009; Straka 2011; Fu 2013). Unexplained RM demonstrate not only intrauterine immunological disturbances but taking into consideration immunopathologic mechanisms of rejection of fetal allograf, the group of patients suffering from RM of unknown etiology are probably much more heterogenic in immunologic parametres observed in peripheral blood.

We found the occurrence of different immunological disturbances in 69.1% of Polish patients suffering from primary recurring miscarriage. Our data indicates that apart from autoimmunologic disturbances, alloimmune factors may play an important role in the failure to conceive.

Positive results for anti-thyroid antibodies (both anti-Tg and anti-TPO) were detected in 21.9% of RM women compared to 16.0% of control patients. The association between thyroid antibodies and RM might be explained by a general increase in autoimmunity against the foetal allograft (Prummel *et al.* 2004; Stagnaro *et al.* 2004). Elevated anti-thyroid antibodies, anti-Tg, and anti-TPO, were found to be prevalent in a group of patients with late pregnancy loss ($p \leq 0.01$, 95% CI=21.3–52.3). There are reports recommending serial thyroid function tests in women with RM, particularly – when pregnancy is established (Kim *et al.* 2010). Our results lead us to agree with Poppe *et al.* who recommend screening pregnant women for anti-thyroid antibodies and for thyroid function. Thyroid replacement with levothyroxine in euthyroid women with thyroid peroxidase antibodies (TPO) increased the proportion of women who attain a live birth beyond 34 weeks of gestation by at least 10% (Poppe *et al.* 2003).

In this study, anti-nuclear antibodies (ANA) were found almost twice as frequently in RM patients as in healthy women (18.7% vs. 10.0%). In previous investigations, we noticed that the high incidence of ANA is characteristic for women with RM of unknown etiology, but not for physiologic pregnancy (Malinowski *et al.* 1994). ANA was found in women with endometriosis and idiopathic fertility (70% and 63% of cases, respectively) (Malinowski *et al.* 1995). The data suggest that ANA may be used as a marker of pregnancy failure. The analysis based on cluster analysis revealed a strong similarity between anti-nuclear (ANA) and anti-thyroid antibodies (ATA). According to Khashan *et al.* (Khashan *et al.* 2011), the incidence of autoantibodies may conclude the role of foetal microchimerism in the development of subsequent maternal autoimmune disease.

Anticardiolipin antibodies were found in 16.1% and lupus anticoagulant was detected in 13.5% of serum samples from RM patients, similarly as reported by other authors (Branch *et al.* 1997; Heilmann *et al.* 2001; Marai *et al.* 2004; Meroni *et al.* 2004; Roye-Green *et al.* 2011; Kovacs *et al.* 2012). Antiphospholipid (APS) syndrome has an outstanding importance in leading to infertility, but the clinical picture needs to be assessed by an immunologic test fulfilling the criteria. In our data primary APS were stated in 25 patients with RM, taking into consideration the cilinical and laboratory tests. In other studies analysing the frequency of antiphospholipid syndrome and pregnancy loss in Polish women (Skrzypczak *et al.* 2011) 3.69% of 352 women met the criteria of the antiphospholipid syndrome. The frequency of APS in women with early and late pregnancy losses were 1.33% and 6.25%, respectively. The results of our investigations concerned the group of patients suffering from RM who took part in the study with negative LCT and lack of blocking proliferative activity in mixed lymphocyte reaction test (MLR) and rather indicate the significance of latent autoimmune disease demonstrating in infertility.

Anti-placental antibodies (APA) were found in 14.8% of RM patients but only in 4.0% of control patients. Their clinical prognostic values need further investigation.

Anti-sperm antibodies (ASA) were found in 22.6% of serum samples from RM patients, while in other reports (Kamieniczna *et al.* 2004) only 4.1% of the serum samples from infertile women tested positive for ASA. In the control fertile women, 8.0% of the serum samples tested positive for ASA. Future studies will be undertaken to provide more accurate information regarding ASA frequency and its effect on fertility status.

In positive serum samples, the number of immunological disruptions increased according to the number of miscarriage, 55.9% of RM patients with 3 pregnancy loss tested positive for immunological disturbances, compared with 66.6% of patients who underwent 5 and more abortions similar as reported in previous texts (Kruse *et al.* 2002; Pfeiffer *et al.* 2001). Among these patients, positive levels of TNFα produced by peripheral blood lymphocytes (30.0%, p=0.05) was the highest risk factor. Gharesi-Fard B. *et al.* reported similar results. The results of our investigation confirm the role of TNFα in RM and propose the assessment of TNFα production as a valuable prognostic parametre for the prediction of pregnancy. Early pregnancy loss were found to be most frequent between 7 and 8 weeks of gestation.

The heterogeneity of immunological risk factors in patients with RM shown in this study and in other reports indicate that autoimmune conditions correlate with alloimmunologic disturbances. In conclusion, our data indicate the usefulness of detecting alloimmune factors (LCT test, blocking proliferative activity in MLR test, Th1 cytokine activity in peripheral blood) as well as autoantibodies in patients with RM of unknown etiology. Women with recurrent miscarriage should have prompt access to Early Pregnancy Assessment Unit supporting their care and management.

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