Characteristics of immune cell changes before and after immunotherapy and their clinical significance in patients with unexplained recurrent spontaneous abortion

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ABSTRACT. To investigate the characteristics of immune cells before and after immunotherapy and their clinical significance in patients with unexplained recurrent spontaneous abortion (URSA), an analysis of 67 URSA patients, 67 sporadic spontaneous abortion (SA) patients, and 22 normal nonpregnant women (as controls) was conducted. URSA patients underwent immunotherapy using paternal lymphocytes. Peripheral blood from patients and controls was examined for lymphocytes and other markers of immune status. Before the immunotherapy, lymphocyte counts, CD4:CD8 cell ratios, and the relative proportion of natural killer (NK) cells were significantly higher in the URSA patient group than in the SA patient

and control groups (P < 0.05). After the therapy, all of these three measures were decreased, whereas the percentage of T cells was increased, and statistically significant differences before and after the immunotherapy were detected (P < 0.05). Therefore, the immune system appears to be activated in the URSA patients, and the abnormal immunologic state in the URSA patients is more severe than in the SA patients. The alterations in T and NK cells may be involved in the etiopathogenesis of URSA. Lymphocyte immunotherapy appears to be an effective treatment for URSA patients.

Key words: Immune cell; Unexplained recurrent spontaneous abortion

INTRODUCTION

The mechanisms underlying maternal-fetal immune tolerance have continued to be a focus of researchers because the maternal immune system provides nutrition and protection to the fetus rather than attacking it. An embryo that expresses paternal antigens may be considered a semi-allograft to the mother. Successful pregnancy depends on the immunologic status of the woman and her immunoregulatory compatibility with the embryo (Aluvihare et al., 2005; Guleria and Sayegh, 2007; Chen et al., 2012).

If the mechanism of immune tolerance is ablated, spontaneous abortion and other pathological effects may result. Recurrent spontaneous abortion (RSA) refers to the loss of pregnancy for ≥2 times in patients before 20 weeks of gestation or a fetal weight of <500 g (Mei et al., 2010; Beaman et al., 2012). RSA has been estimated to affect 5% of women of reproductive age. In fact, the incidence of RSA is even higher because of the exclusion of subclinical and underdiagnosed RSAs in these statistics (Stephenson and Kutteh, 2007).

RSA has a complex etiology, with factors thought to include chromosomal, anatomic, endocrine, and autoimmune abnormalities, as well as infections of the reproductive tract. In at least 35-44% of patients, the factors inducing abortion are still unclear; these particular cases are now referred to as unexplained recurrent spontaneous abortion (URSA). However, it has recently been shown that URSA has features of an alloimmune disease associated with failure of the fetal-maternal immunologic tolerance (Christiansen et al., 2004; Kwak-Kim et al., 2010; Leber et al., 2011).

It has been reported that the mixed lymphocyte reaction blocking factor (MLR-Bf) plays a fundamental role in immune tolerance during pregnancy. MLR-Bf is present in the IgG3 fraction during pregnancy but absent in women with RSA. Following immunotherapy, IgG3 is detectable in women with RSA, playing an important role in the maintenance of pregnancy. However, reported changes in T, B, and natural killer (NK) cells during immunotherapy are limited, and few studies have assessed these differences in immune cell counts between URSA and sporadic spontaneous abortion (SA) patients.

Based on these findings, the purpose of this study was to determine the characteristics of immune cells and their clinical significance in MLR-Bf-negative URSA patients before and after immunotherapy. We enumerated lymphocytes, T cell subsets, B cells, and NK cells in URSA patients, SA patients, and healthy women to provide a clinical basis for disease prevention and control.

MATERIAL AND METHODS

Subjects

Sixty-seven URSA patients were recruited from the Department of Gynecology and Obstetrics at Memorial Hospital of Sun Yat-sen University between May 2008 and April 2011. All patients met the following conditions: early SA ≥2 times (during the period of 6-12 weeks of gestation), MLR-Bf negative, and normal karyotypes in both partners. Of these patients, we excluded those having infectious, endocrine, metabolic, anatomic, or autoimmune disease. At least 3 months had elapsed since the last SA.

Twenty-two healthy, non-pregnant women who underwent physical examinations in our hospital during the same period were randomly selected and recruited into the control group. All of the subjects in the control group had had at least 1 live birth and no history of abnormal pregnancies or abortions.

This study was approved by the Ethics Review Board of the Second Affiliated Hospital of Sun Yat-sen University. The study had been explained in detail to all study participants and all patients provided informed consent before this study was conducted.

Medical histories and physical examination

For each patient a thorough medical and obstetric history was obtained, and a physical examination was performed during the first visit to our department. Examinations were performed by gynecologists with >5 years of clinical experience.

Specimen collection and clinical treatment

Before lymphocyte immunotherapy, 3 mL peripheral blood was obtained from patients in the 3 groups on the 14th day of the menstrual cycle and immune cells were detected and analyzed. The URSA patients then underwent lymphocyte immunotherapy as described in the following: 5 x 10^7 cells/mL paternal lymphocytes were dissolved in 2 mL sterile normal saline, and the cell suspension was injected intradermally (Pandey and Agrawal, 2004). The patients underwent this treatment once every 4 weeks, for a total of 4 treatments to complete the course. After the therapy, peripheral blood was again obtained from the URSA patients.

Detection methods

Heparin was added to each blood sample to prevent coagulation. Gradient centrifugation and the Ficoll-Hypaque method was used to separate peripheral blood mononuclear cells. The cell suspensions were treated with the following antibody combinations: fluorescein isothiocyanate (FITC)-conjugated anti-CD45, phycoerythrin (RD1)-conjugated anti-CD56, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-conjugated anti-CD19, and phycocyanin 5 (PC5)-conjugated anti-CD3; alternatively, they were treated with FITC-conjugated anti-CD45, RD1-conjugated anti-CD4, EDC-conjugated anti-CD8, and PC5-conjugated anti-PC5. Stained cells were analyzed using a Beckman Coulter FC 500 flow cytometer and the

Cell Quest software to sort and calculate cell subsets. We determined the total number of lymphocytes, T cell subsets (CD4+ and CD8+ T cells), and the percentage of T, B, and NK cells.

Statistical analysis

The SPSS13.0 statistical software was used to perform statistical analyses. Data are reported as means ± standard deviation (SD). Values were tested for normal distribution using the Shapiro-Wilk test. As the parameters were normally distributed, we applied two independent sample *t*-tests for comparisons between groups. The paired *t*-test was used for intergroup comparisons. A $P < 0.05$ was considered to be statistically significant.

RESULTS

General conditions

No statistically significant differences in age, menstrual cycle, gravidity, nationality, place of residence, or BMI were detected among the URSA, SA, and control groups (Table 1).

Table 1. General conditions of the groups studied.

Group	URSA group	SA group	Control group	URSA *vs* SA	P	
					URSA *vs* control group	SA *vs* control group
Age (years)	30.28 ± 4.12	30.00 ± 4.95	29.67 ± 3.29	0.063	0.114	0.062
Menstrual cycle (days)	31.33 ± 5.02	33.61 ± 5.78	31.00 ± 3.94	0.343	0.547	0.14

Data are reported as means ± SD. URSA = unexplained recurrent spontaneous abortion; SA = sporadic spontaneous abortion.

Pregnancy phases of spontaneous abortions

Of the 67 URSA patients, 34, 26, and 7 had 2, 3, and >3 SAs, respectively. In total, 182 SAs had occurred among the 67 patients. The status of embryos was determined based on ultrasonogram reports. The reports described the embryos as empty gestational sacs, fetal poles without cardiac activity, a fetal heart beat that was initially detected, but then lost, or as chemical pregnancies. As shown in Table 2, in 9 cases, embryos were described as fetal poles without cardiac activity and 31 as empty gestational sacs, descriptions that indicated spontaneous abortion had occurred before the formation of the cardiac system.

Table 2. Pregnancy phases of spontaneous abortion.

Factors	URSA group	SA group
Have no idea	106	35
Empty gestational sac	31	11
Fetal pole	9	12
Cardiac activity	26	6
Chemical pregnancy	10	3

Embryo conditions were determined by retrospective data, and "have no idea" were those in which the patient did not know or could not recall the reasons given to their previous pregnancy losses; empty gestational sac means no fetal pole or fetal cardiac activity detectable by ultrasound; fetal pole means an embryo without fetal cardiac activity by ultrasound; cardiac activity means that a fetal heart beat was initially detected but was lost. For abbreviations, see legend to Table 1

Comparison of immune cells

Approximately 20-40% of all white blood cells are lymphocytes. In this study, the lymphocyte count in the URSA group before immunotherapy was significantly higher than in SA and control groups ($P < 0.05$); however, the lymphocyte count in the URSA group after therapy was lower than before the therapy ($P < 0.05$). The lymphocyte count in the URSA group after therapy was similar to that in SA and control groups.

T cells originate from stem cells in hematopoietic tissue and undergo differentiation in the thymus triggered by thymopoietin, and some studies have reported that T cells exert a greater effect in patients with URSA (Yang et al., 2010). Our data showed that the URSA group before therapy had the lowest percentage of T cells compared with SA and control groups. A statistically significant difference was detected between the URSA group before therapy and the control group, but no such difference was detected between the URSA group before therapy and the SA group. The percentage of T cells in the URSA group after the therapy was higher than the T cell percentage before the therapy (71.46 ± 7.29 *vs* 67.77 ± 8.45%, respectively; $P < 0.05$).

Moreover, before immunotherapy, the proportion of CD4+ T cells in the URSA group was higher than in SA and control groups. A statistically significant difference was detected between the URSA group before therapy and the SA group ($P < 0.05$), whereas no statistically significant differences were detected between the URSA group after therapy and the SA group, and between the control and SA groups.

In addition, no significant differences in the percentage of CD8+ T cells were detected before and after therapy in the URSA group, and between the URSA and the SA and control groups.

The CD4:CD8 cell ratio was significantly higher in patients with URSA than in subjects of the SA and control groups ($P < 0.05$). After immunotherapy with paternal lymphocytes, the ratio of CD4:CD8 cells was decreased: the CD4:CD8 ratio before therapy was 1.86 ± 0.55 and after therapy 1.69 ± 0.50 ($P < 0.05$).

No difference in the percentage of B cells was detected before and after therapy in the URSA group, and between the URSA group and the SA and control groups.

The proportion of NK cells was significantly higher in the URSA patients than in SA patients and in the subjects of the control group ($P < 0.05$). In addition, a statistically significant difference in the proportion of NK cells was detected between the SA and control groups. Immunotherapy decreased the proportion of NK cells to levels that were lower than in the SA group ($P < 0.05$), but NK cell levels after immunotherapy remained higher than in the control group (Table 3; Figures 1 and 2).

DISCUSSION

Pregnancy, in immunologic terms, is often considered to be a conspecific, semi-heterologous implantation process in which the semiallogeneic fetus is protected from attack by the maternal immune system. Once the maternal-fetal immune tolerance is broken, adverse pregnancy outcomes result (Jin et al., 2011).

Several studies have suggested that SA and RSA are alloimmunity diseases associated with failure of maternal-fetal immunologic tolerance (Bulletti et al., 1996; Saito et al., 2011). Because embryo attachment and early implantation are exquisitely controlled by the local and

Table 3. Comparison of immune cells.

Testing item	URSA group		SA group	Control group	P					SA vs control
	Before therapy	After therapy			URSA before therapy vs SA	URSA after therapy vs SA	URSA before therapy vs URSA after therapy	URSA before therapy vs control	URSA after therapy vs control	
Lymphocytes (10^9/L)	2.26 ± 0.62[a,c,d]	2.05 ± 0.49[b]	2.03 ± 0.46[b]	1.93 ± 0.54[b]	0.001	0.100	0.012	0.000	0.23	0.088
T cells (%)	67.77 ± 8.45[c,d]	71.46 ± 7.29[b]	69.24 ± 8.26	73.32 ± 6.11[b]	0.318	0.105	0.000	0.003	0.272	0.172
CD4 T cells (%)	40.16 ± 8.11[a]	40.00 ± 5.93[a]	37.05 ± 5.25[b,c]	39.56 ± 5.90	0.015	0.030	0.094	0.197	0.699	0.812
CD8 cells (%)	23.75 ± 5.18	25.04 ± 5.72	24.31 ± 9.36	25.27 ± 3.97	0.643	0.611	0.105	0.188	0.167	0.06
CD4/CD8	1.86 ± 0.55[a,c,d]	1.69 ± 0.50[b]	1.59 ± 0.45[b]	1.56 ± 0.39[b]	0.003	0.836	0.036	0.001	0.699	0.554
B cells (%)	13.19 ± 4.31	14.01 ± 4.35	13.79 ± 5.09	12.56 ± 3.36	0.182	0.08	0.136	0.232	0.437	0.631
NK cells (%)	17.46 ± 7.77[a,c,d]	11.98 ± 4.76[a,b]	14.98 ± 6.65[b,c,d]	9.84 ± 3.07[a,b]	0.045	0.003	0.000	0.000	0.099	0.001

Values are reported as means ± standard deviation. [a]P < 0.05 versus sporadic abortion group. [b]P < 0.05 versus the URSA group before therapy. [c]P < 0.05 versus the URSA group after therapy. [d]P < 0.05 versus the control group. Difference considered to be statistically significant at P < 0.05. For abbreviations, see legend to Table 1

Peripheral blood immunocytes records
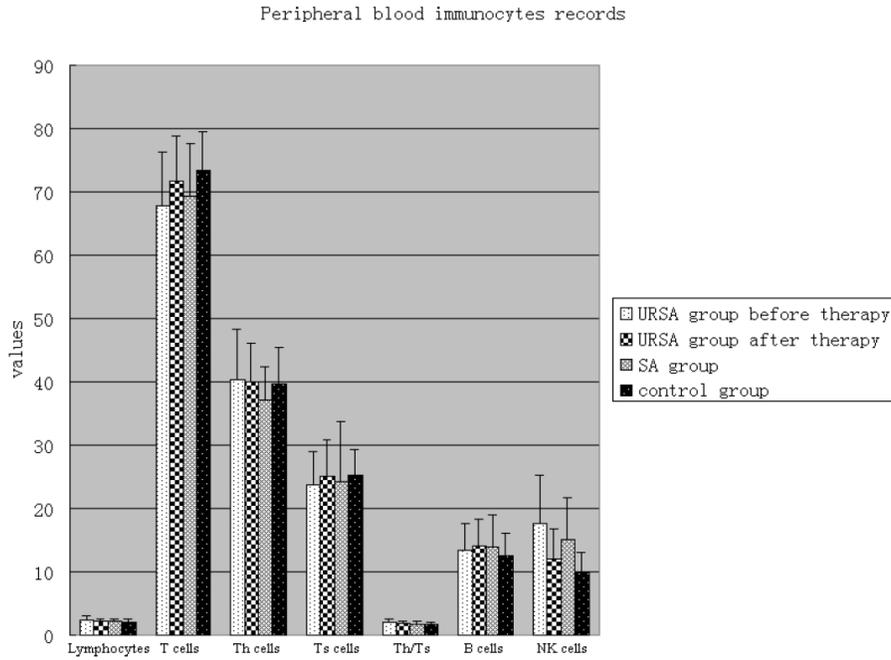
Figure 1. Peripheral blood immunocyte records. Before immunotherapy, the lymphocyte count, the CD4:CD8 cell ratio, and the proportion of natural killer (NK) cells were higher in unexplained recurrent spontaneous abortion (URSA) patients compared with sporadic spontaneous abortion (SA) and control groups (P < 0.05). In the URSA group after immunotherapy, the lymphocyte count, the CD4:CD8 cell ratio, and the proportion of NK cells were decreased (P < 0.05). Moreover, after immunotherapy the percentage of NK cells in the URSA group was sifnificantly lower than in the SA group (P < 0.05). Data are reported as means ± SD.
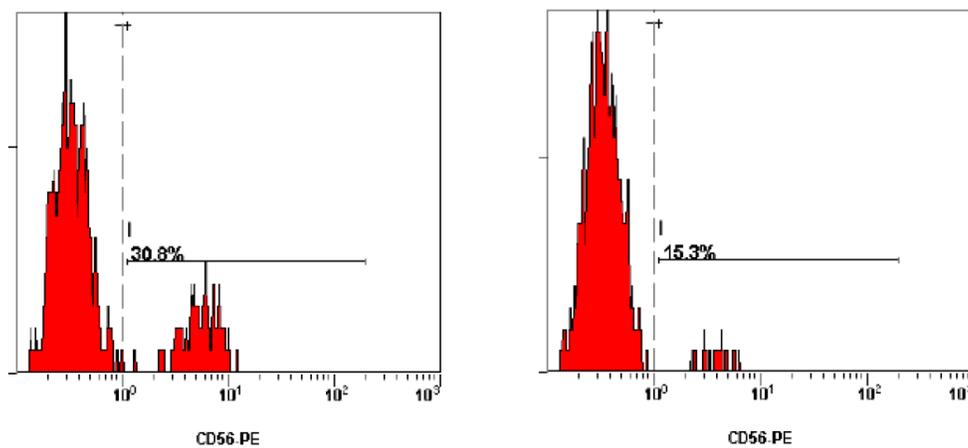
Figure 2. Flow cytometry of NK cells in URSA patients before and after therapy. The URSA patient was 31 years old and had two spontaneous abortions. The menstrual cycle was 28 days. Before therapy, the percentage of NK cells was 30.8%. After lymphocyte immunotherapy, the percentage of NK cells was 15.3%.

systemic immune responses, immune-related pregnancy failures likely occur early in gestation. It has been reported that during the implantation period, a significant number of embryos are lost, and less than half of clinically established pregnancies eventually carry to full term without obstetric complications (Kwak-Kim et al., 2010). In this study, it was shown that most SAs and RSAs occurred soon after implantation, before the development of the fetal cardiac system as evidenced by the description of fetuses with empty gestational sacs and fetal poles without fetal cardiac activity.

Recent studies have shown that a variety of immune cells and cytokines play a major role in allograft rejection (Wilczyński, 2006). It is believed that lymphocytes are responsible for immunologic memory. Thus, repeated contact with an antigen elicits an accelerated and increased response (Danilova, 2012). In the current study, the lymphocyte count was higher in the URSA group before immunotherapy than in SA and control groups. After lymphocyte immunotherapy, the lymphocyte count was significantly decreased in the URSA group. These results support the hypothesis that the immunologic state is different between URSA and SA patients. The abnormal lymphocyte count in URSA patients was more severe than in SA patients. Indeed, lymphocyte immunotherapy alters the level of peripheral blood lymphocytes.

Lymphocytes comprise small and large lymphocytes, with the former consisting of B cells and the latter of large granular lymphocytes including NK cells. T cells, on the other hand, are involved in cell-mediated immunity and are divided into different subsets on the basis of expression of multiple antigens. For example, CD4+ cells are helper/inducer T lymphocytes, and CD8+ cells belong to the cytotoxic/suppressor T lymphocyte lineage. CD4+ lymphocytes act as helper cells and increase the immune response, whereas CD8+ lymphocytes suppress it.

Recent evidence indicates that the CD4:CD8 ratio is low in diseases that are associated with reduced immunologic reactivity, whereas it is high in diseases that are associated with increased immunologic reactivity, such as autoimmune diseases or renal allograft rejection (Margolick et al., 2006; Hussein et al., 2008). During normal pregnancy, the percentage of CD4+ cells is decreased and the percentage of CD8+ cells and the CD4:CD8 ratio is decreased (Makrydimas et al., 1994; Luppi et al., 2002).

In this study, the percentage of CD4+ cells and the CD4:CD8 ratio was higher in the URSA group before immunotherapy than in SA and control groups. Moreover, the percentage of CD8+ cells was lower in the URSA group before therapy than in SA and control groups. The percentage of CD4+ cells and the CD4:CD8 ratio decreased and the percentage of CD8+ cells increased following therapy. Therefore, we hypothesize that in URSA patients the CD4+ cells mediate cellular immunity, with an increase in the number enhancing immune function of maternal cells and increasing the embryonic immune rejection response. The immunologic dysfunction cannot be restored to normal levels spontaneously. Lymphocyte immunotherapy alters the proportions and functions of T cell subsets. Some of these alterations may be beneficial for maintaining pregnancy (Bartha et al., 2000).

B lymphocytes are involved in humoral immunity and respond to pathogens by producing large quantities of antibodies, which neutralize foreign objects. A mature B lymphocyte is activated by binding antigen to a specific cell-surface receptor. This induces cell proliferation, resulting in many cell clones specific to that antigen. These cells then differentiate and begin to secrete immunoglobulins (Igs). About 10 years ago, it was reported that the percentage of B lymphocytes was significantly higher in women with unexplained habitual miscarriages than in healthy women (Darmochwal-Kolarz et al., 2002). Moreover, the proportion of B cells (CD19+) increased during the first trimester of pregnancy in RSA women compared with healthy pregnant

controls (Jablonowska et al., 2002). However, in 2011, Shankarkumar et al. investigated the autoantibody profile and B cells among RSA patients and healthy pregnant women from Mumbai in western India. These results revealed 34% positivity of all autoantibodies tested among RSA patients. The lower expression of CD19 (B cells) was observed in 18% of the patients, whereas increased expression of CD19 cells was observed in 12% of the patients compared to the controls. In this study, the percentage of B cells in the URSA group was similar to that in the SA group. These data suggest that in the etiopathogenesis of spontaneous pregnancy loss, the level of autoantibodies may be more important than the percentage of B cells.

As nonspecific immunocompetent cells, NK cells have cytotoxic effects against a variety of target cells. In normal conditions, NK cells are in a resting state with low cytotoxic activity. During a normal pregnancy, embryos can be considered a semi-allograft with maternal immune rejection in an inhibitory state. When there are significant changes in NK cell proliferation and cytotoxic activity, the dynamic immune equilibrium between mother and fetus is compromised, possibly leading to pregnancy loss.

Some authors have suggested that the number and ratio of NK cell subtypes may be used as an effective index in URSA patients (Bansal et al., 2012). The peripheral blood NK cells reflect changes in decidual NK cells in women with RSAs (Park et al., 2010). In this study, URSA patients had a higher percentage of NK cells and differences in the peripheral blood NK cells were observed between the SA and the URSA patients.

Lymphocyte immunotherapy involves using the lymphocytes, white blood cells, and mononuclear cells from donors as immunogens in immune RSA patients. These cells induce production of antibodies against human leukocyte antigen and MLR-Bf, thus preventing recognition of embryonic paternal antigens and destruction by the maternal immune system. In this way, pregnancy is maintained (Adachi et al., 2003; Pandey and Agrawal, 2004). In this study, the immunotherapy using paternal lymphocytes decreased the lymphocyte count, the CD4:CD8 cell ratio, and the proportion of NK cells. Thus, as has been previously reported by Pandey and Agrawal (2004), these results suggest that immunization of the patients with paternal leukocytes corrects the dysregulated anti-fetal immune response in the URSA.

In conclusion, URSA is associated with abnormalities of the cellular immune system. The immunologic alterations of T and NK cells may be involved in the etiopathogenesis of URSA. Lymphocyte immunotherapy seems to be an effective treatment for URSA patients with immune abnormalities.

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