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Immunological evaluation of patients with recurrent abortion

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Abstract

In a prospective study, we performed immunological tests in patients with recurrent abortion. Nine couples with two or more fetal losses of no apparent cause were selected as the patient group, and nine volunteer couples with at least two children and without a history of abortion were used as controls. The frequency of major histocompatibility complex (human leukocyte antigens, HLA) antigen sharing was determined by serological methods, antipaternal antibodies by microlymphocytotoxicity, lymphocyte phenotypes (CD4, CD8, CD19, CD16, CD56 and HLA-DR positive cells) by flow cytometry and natural killer (NK) cytotoxicity by ^{51}Cr release. NK activity was correlated to the degree of HLA-C sharing and to the percentage of CD16+ and CD56+ cells and to progesterone levels measured by radioimmunoassay. No difference in class I or class II HLA antigen sharing was detected between couples with and without recurrent abortion. Antipaternal antibodies were not found in the serum of any woman of the study. A higher absolute number of CD8+ cells ($P = 0.01$) and a trend to increased CD19+ cells ($P = 0.05$) were observed among patients. NK activity did not differ between the two groups when expressed as specific cytotoxicity and it was reduced among patients with recurrent abortion when expressed as lytic units/10^7 cells ($P = 0.04$). There was correlation between NK activity and

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the percentage of CD16+ and CD56+ cells but not with progesterone levels in patients with recurrent abortion. Our data suggest that an increased NK activity may not play a role in the occurrence of repeated abortion. On the other hand, an increase in circulating CD8+ T cells was observed in patients suggesting that antifetal cytotoxicity in recurrent abortion may be mediated by T cells and not by NK cells.

Keywords: Recurrent abortion; Natural killer activity; Lymphocyte subsets; Human leukocyte antigens; Progesterone

1. Introduction

The reproductive success of the human species continues to be an immunological enigma. Recurrent abortion, classically defined as three or more consecutive spontaneous fetal losses before 20 weeks of gestation or before fetal weight reaches 500 g (Edmonds et al., 1982), is a complication of pregnancy that may affect up to 2% of couples who try to have children. The disorder is called primary or secondary depending, respectively, on the absence or the presence of a viable live fetus preceding the sequence of abortions (Stirrat, 1990). Among clinically recognized pregnancies, 15–20% suffer fetal loss, most of them before 20–22 weeks of gestation (Mishell, 1993; Zinaman et al., 1996).

Some studies have suggested that reproductive success may be due to a disparity in human leukocyte antigens (HLA) between husband and wife leading to maternal-fetal intolerance (Komlos et al., 1977; Beer et al., 1981), although this concept is still controversial. Nevertheless, allogenic cells from the husband bearing disparate HLA molecules from the fetus have been used to vaccinate patients with recurrent abortion since 1981, with variable results (Mowbray et al., 1985; Smith and Cowchock, 1988; Ober et al., 1999). Immunological alterations associated with repeated abortion that are potentially reversible by immunotherapy are the appearance of lymphocytotoxic antibodies against paternal antigens, changes in lymphocyte subpopulations, and abnormal natural killer (NK) activity (Makida et al., 1991). However, the definitive contribution of these and other immunological factors to the pathogenesis of repeated abortion is unknown.

The objective of the present study was to determine the role of shared major histocompatibility complex antigens (human HLA system), of lymphocytotoxic antibodies against paternal antigens, of lymphocyte subpopulations (B, T and NK cells), and of NK activity in patients with recurrent abortion compared to a control group of fertile women.

2. Material and methods

The study group consisted of nine couples with recurrent abortion of no apparent cause, compared to a control group of nine fertile couples. The couples with recurrent abortion were seen at the Infertility Clinic of the Department of Gynecology and Obstetrics of the School of Medicine of Ribeirão Preto, University of São Paulo, with a history of two or more previous spontaneous and consecutive abortions. They underwent initial investigations which showed normal chromosomal analysis of both partners, negative tests for anticardiolipin antibodies and lupus anticoagulant, no evidence of structural uterine anomaly by pelvic ultrasonography, hysterosalpingogram and hysteroscopy, normal values of follicle stimulating hormone, luteinising hormone, prolactin, thyroid function tests and endometrial biopsy. Patients with secondary abortion and other known causes of repeated abortions were excluded. The control group consisted of volunteer couples with a history of two or more term pregnancies, without abortion, aged < 40 years, and with no apparent diseases. Immunological tests were performed outside the gravidic–puerperal cycle and the subjects were not using any medication. The Committee of Ethics in Research of the Ribeirão Preto University Hospital approved the investigation.

All cell tests comparing patients and controls were carried out during the second phase of the menstrual cycle using fresh lymphocytes isolated on a Ficoll–Hypaque gradient and then submitted to monocyte removal by adhesion to glass plates. In addition, lymphocyte subsets from patients were compared between the first and second phase of the menstrual cycle.

2.1. Human leukocyte antigens typing

HLA class I (A, B and C) and class II (DR and DQ) antigen typing was performed in the couples by the complement-dependent microlymphocytotoxicity method using commercial anti-HLA sera (Biotest, Germany). The same microlymphotoxicity method was used for the detection of lymphocytotoxic antibodies in maternal serum directed against paternal antigens.

2.2. Lymphocyte subpopulations

The percentage of peripheral blood lymphocytes positive for the antigens CD4, CD8, CD19, CD16, CD56 and HLA-DR were determined by flow cytometry using monoclonal antibodies, a FACSort flow cytometer and the Lysis II analysis program (Becton Dickinson, San Jose, CA). The

absolute numbers of each phenotype were obtained by multiplying the percentages by the total number of lymphocytes determined in an automated hematological cell counter.

2.3. Natural killer activity

NK activity was determined by a ^{51}Cr release assay using as targets the K562 tumor cell line labeled with 3.7 Mbq (100 μCi) of $Na_{251}CrO_4$ (specific activity: 370 MBq/ml Cr) obtained from the Atomic Energy Institute of Sao Paulo. The cytotoxic assay was set up on polystyrene 96-well U-shaped culture plates in triplicates, at the following effector/target cell ratios: 320:1, 80:1, 20:1, 5:1, and 1.25:1. After 4 h of incubation, ^{51}Cr release was measured in a gamma counter (Cobra Auto-Gamma, Packard, Meridien, USA). NK activity was expressed as specific cytotoxicity (observed cpm − spontaneous cpm/maximum cpm − spontaneous cpm) and as the number of 40% lytic units, $LU/10^7$ cells.

2.4. Progesterone levels

Serum progesterone was measured by solid phase radioimmunoassay without previous extraction using commercial DPC kits (Diagnostic Products Corporation, LA) with a sensitivity of 0.03 ng/ml.

2.5. Statistical analysis

Fisher's exact test was used to estimate HLA antigen sharing. Comparison of related groups was performed by the Wilcoxon test and comparison of unrelated groups was performed by the Mann–Whitney test. Correlations were determined by the Spearman test. Two-tailed tests were used and the level of significance was set at $P < 0.05$.

3. Results

When class I and II HLA antigen typing of couples with recurrent abortions were compared with controls, we could not detect any significant difference in the number of antigens shared by the two groups (Table 1). This was true both for all antigens studied as a whole and for class I and class II antigens separately. In general, there was more sharing of HLA antigens among controls than patients, with HLA-C being the exception, but the numbers are too small to reach statistical significance. The absolute numbers/mm^3 of CD16 + and CD56 + lymphocyte subpopulations and NK activity expressed

as the number of LU40%/10^7 cells in couples sharing 0 vs. 1 or 2 HLA-C antigens are shown in Table 2. No significant difference in NK phenotypes or function could be detected between patients and controls with different degrees of HLA-C sharing.

Cross-reactions between the serum of the women and the lymphocytes of the partner to detect antipaternal antibodies were negative in all subjects of both groups.

The percent values of peripheral blood CD4 + , CD8 + , CD19 + , CD16 + , CD56 + and HLA-DR + observed between the first and second phase of the menstrual cycle in patients with recurrent abortion were not found different for any of the cell phenotypes studied (results not shown). When the patients were compared to the controls in the second phase of the menstrual cycle, no differences were observed, although circulating CD8 +

Table 1
Frequency of HLA class I and II antigen sharing in patients with recurrent abortion compared with controls

HLA antigens	No. of antigens shared	Patients (n, %) $n = 9$	Controls (n, %) $n = 9$	P*
A	0	6 (66.7)	3 (33.3)	0.35
	1 or 2	3 (33.3)	6 (66.7)	0.35
B	0	7 (77.8)	7 (77.8)	1.00
	1 or 2	2 (22.2)	2 (22.2)	1.00
C	0	2 (22.2)	5 (55.5)	0.33
	1 or 2	7 (77.8)	4 (45.5)	0.33
DR	0	6 (66.7)	4 (45.5)	0.64
	1 or 2	3 (33.3)	5 (55.5)	0.64
DQ	0	1 (11.1)	0 (0)	1.00
	1 or 2	8 (89.9)	9 (100)	1.00
A, B, C	0	1 (11.1)	0 (0)	1.00
	1	5 (55.5)	6 (66.7)	1.00
	2 or more	3 (33.3)	3 (33.3)	1.00
DR, DQ	0	1 (11.1)	0 (0)	1.00
	1	5 (55.5)	4 (45.5)	1.00
	2 or more	3 (33.3)	5 (55.5)	0.64
A, B, C, DR, DQ	0	0 (0)	0 (0)	1.00
	1	1 (11.1)	0 (0)	1.00
	2 or more	8 (89.9)	9 (100)	1.00

* Fisher exact test.

Table 2
Phenotypes and functional activity of NK cells related to the number of HLA-C antigens shared by couples with recurrent abortion (patients) or healthy controls

Cellular parameter	No. of HLA-C antigens shared	Patients, $N = 9$	Controls, $N = 9$	P*
NK activity (LU40%/10^7 cells)	0	9.6	13.7	0.38
	1 or 2	6.3	14.9	0.23
CD16+ (cells/mm^3)	0	211	192	0.86
	1 or 2	134	190	0.79
CD56+ (cells/mm^3)	0	257	230	0.57
	1 or 2	169	251	0.53

* Mann–Whitney test comparing patients vs. controls.

cells showed a trend to be more elevated in patients with recurrent abortion ($P = 0.06$).

The absolute numbers/mm^3 of CD4+, CD16+, CD56+ or HLA-DR+ lymphocyte subpopulations were not different between patients with recurrent abortion and fertile controls. In contrast, the number of CD8+ cells was significantly increased ($P = 0.01$) and CD19+ cells tended to be increased ($P = 0.05$) in patients compared to controls (Table 3).

Evaluation of specific cytotoxicity of NK cells at the effector/target cell ratio of 320:1 between the two groups showed a tendency to lower NK activity in the patients with recurrent abortion (46 vs. 54%, $P = 0.06$). When NK activity was expressed as the number of 40% LU/10^7 cells a median of 6.3 was found for the patients with recurrent abortion and of 13.7 for the control group ($P = 0.04$) (Fig. 1).

Table 3
Absolute numbers/mm^3 of lymphocyte subpopulations in patients with recurrent abortion and in the control group during the second phase of the menstrual cycle

Cellular phenotypes	Recurrent abortion median (Q1–Q3) $n = 9$	Controls median (Q1–Q3) $n = 9$	P value*
CD4+	1199 (1050–1265)	1143 (1073–1160)	0.79
CD8+	587 (525–690)	448 (242–448)	0.01
CD19+	215 (188–236)	182 (151–185)	0.05
CD16+	142 (125–166)	192 (92–219)	0.85
CD56+	169 (127–178)	230 (111–273)	0.79
HLA-DR+	196 (151–209)	175 (145–206)	0.66

* Mann–Whitney test.

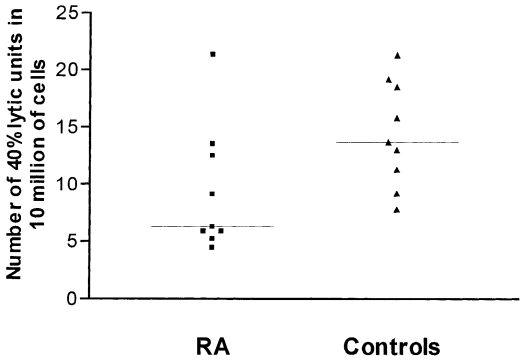

Fig. 1. NK activity in patients with recurrent abortion (RA) and controls expressed by the number of 40% LU in 10 million of cells. Median were represented by horizontal bars. Comparison made by the Mann–Whitney test ($P = 0.04$).

There was a positive correlation between NK activity and the proportion of CD16+ and CD56+ lymphocytes in patients with recurrent abortion but no significant correlation between NK activity and progesterone levels in patients with recurrent abortion and in the controls (Fig. 2).

4. Discussion

In the present study, we investigated immunological mechanisms possibly involved in recurrent abortion, which represents an important clinical problem in the reproductive process.

Conflicting results have been reported in the literature with respect to the participation of major histocompatibility (HLA) antigens in the pathophysiology of recurrent abortion. Some investigators have observed an increased sharing of these antigens (Komlos et al., 1977; Beer et al., 1981), others have reported the presence of certain antigens in patients with recurrent abortion such as HLA-DR5, HLA-B18, HLA-DR17, HLA-DQ2 and HLA-DQA1 (Reznikoff-Etievant et al., 1984; Christiansen et al., 1992; Ober et al., 1992), whereas still others have detected no difference between patients with recurrent abortion and a control group of fertile women (Eroglu et al., 1992; Coulan, 1992). Our data agree with the last group of papers and suggest that increased sharing of histocompatibility antigens may not be related to recurrent abortion in this population. Moreover, NK phenotypes and activity were not correlated to a difference in the degree of HLA-C antigen sharing between patients and controls suggesting that NK alloreactivity mediated by HLA-C incompatibility may not play a role in repeated abortion and confirming previous work by Christiansen (1999). Small numbers of patients

precluded an analysis of individual HLA antigens in this study and the appearance of possible statistical significance in some of the variables studied.

Antipaternal antibodies are observed in the mother serum in a proportion of normal pregnancies and are correlated with success of lymphocyte immunization in some studies (Mowbray et al., 1985). The absence of antipaternal cytotoxic antibodies among our patients with recurrent abortions

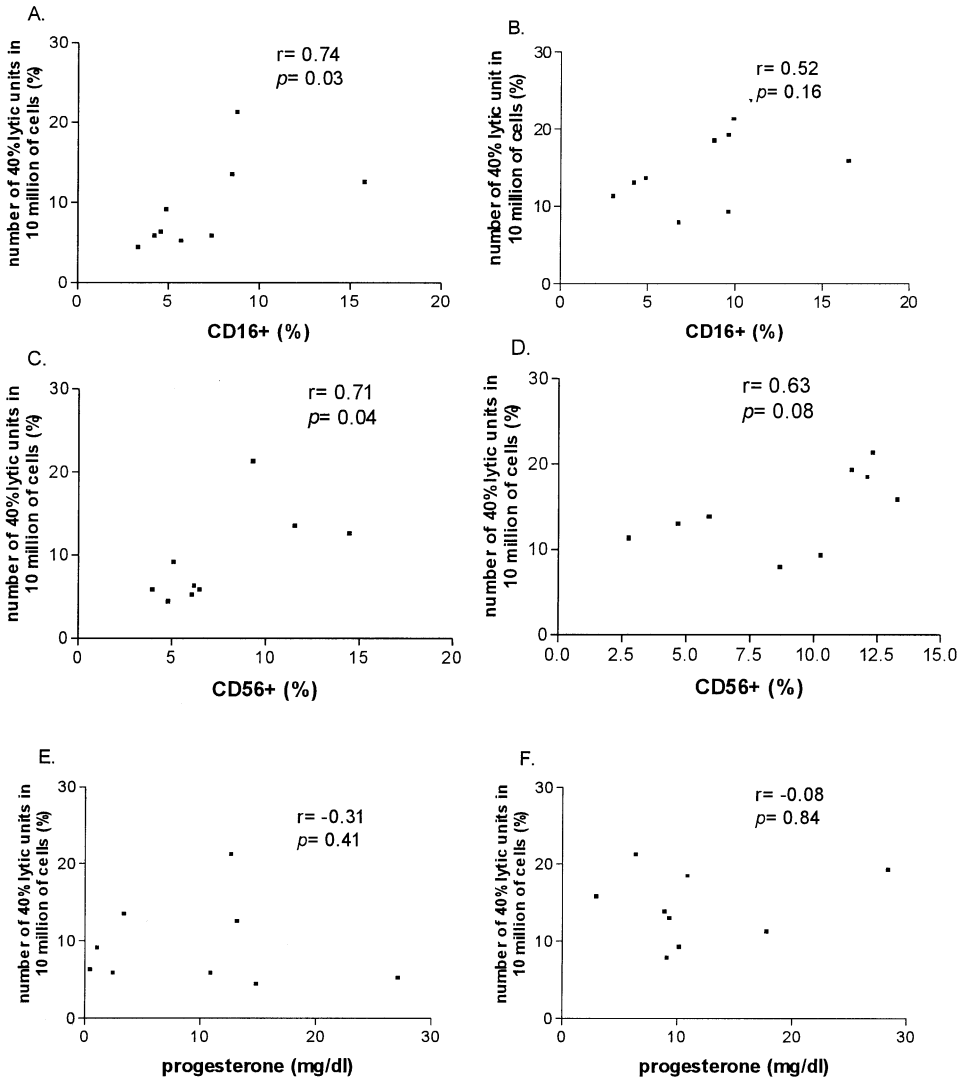

Fig. 2. Correlation by the Spearman test between NK activity and the proportion of (A) CD16+ and (C) CD56+ lymphocytes and (E) progesterone levels in the group of patients with recurrent abortion and (B), (D) and (F) in the controls; *r* and *P* were represented.

may be caused by lack of enough time for antibody formation since the fetal losses occurred during the first trimester of gestation or, alternatively, these antibodies were blocked by anti-idiotypic antibodies formed during gestation (Suciu-Foca et al., 1983). The absence of antipaternal antibodies in the control group suggests that their presence is not imperative for the success of pregnancy as shown by Regan and Braude (1987).

The profile of lymphocyte subpopulations in the peripheral blood was altered in different phases of menstrual cycle of normal women in some studies (Chen et al., 1995) but not in others (Laeopez-Karpovitchs et al., 1993) and changed in women with repeated abortion (Kwak et al., 1995). NK activity is also affected by the menstrual cycle in normal subjects (Souza et al., 2001). In our study, cellular phenotypes were not modified by menstrual cycle in patients with repeated abortion, but they showed an elevation in CD8 + cells and possibly in CD19 + cells in the second phase of the menstrual cycle compared to controls in the absence of any therapeutic intervention. This observation suggests that CD8 + cells of pregnant women may play a cytotoxic role against the embryonic tissues, in contrast to the suppressor role proposed for them by other investigators (Takakuwa et al., 1991). The process of rejection of solid organ transplants is primarily mediated by cytotoxic CD8 + T cells restricted to class I MHC, although the activation of helper T cells is necessary to provide IL-2 needed during the class I MHC-dependent response (Wood, 1994; Garovoy et al., 1994). It is possible that the same mechanism occurs during the process of 'fetal rejection' associated to repeated abortion, according to our results.

NK cells mediate cytotoxic activity independent of previous sensitization and of restriction to MHC antigens. Fougerolles and Baines (1987), in animal models, suggested that spontaneous abortion might be partially mediated by the activity of disordered NK cells. Subsequently, Makida et al. (1991) reported that patients with recurrent abortion who were able to bring a pregnancy to term after immunotherapy with lymphocytes presented a decrease in NK activity compared to the levels before immunotherapy. In addition, Aoki et al. (1995) showed that an elevated preconception NK activity in women with recurrent abortion was a predictive factor for unfavorable gestational evolution. However, we found that untreated patients with recurrent abortion have reduced NK activity compared to the control group, an observation that questions the role of NK activity as a predictive factor of successful pregnancy.

There was a correlation between NK activity and the numbers of CD16 + and CD56 + cells in patients with recurrent abortion but not in control women indicating that the same number of cells may display variable cytotoxic activity. Alternatively, there may be NK cell subpopulations differing from those with CD16 and CD56 cell markers, which were not

studied. The lack of correlation between NK phenotypes and function has been observed in the literature (Whiteside et al., 1990). We did not find any correlation between progesterone levels and NK activity in the second part of the menstrual cycle. Reports in the literature have indicated that progesterone might play a modulatory role on NK activity (Hansen et al., 1992), which was not confirmed by other reports (Sorachi et al., 1993; Szekeres-Bartho et al., 1995) and by our study.

In summary, our data show that absolute numbers of circulating CD8 + cells were elevated in patients during the second half of menstrual cycle compared to the controls and NK activity was decreased. Thus, our results do not support a role for elevated NK cytotoxicity in the mediation of recurrent abortion. Pending on studies with larger series and functional investigations, it is possible that the most important mechanism for fetal loss in recurrent abortion involves cytotoxicity mediated by CD8 + T cells.

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