Results of Immunotherapy for Patients with Unexplained Primary Recurrent Abortions – Prospective Non-Randomized Cohort Study

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Problem
The present study was conducted to examine the efficacy of immunotherapy for unexplained primary recurrent aborters using paternal lymphocytes.

Method of study
Two hundred and twenty-eight recurrent aborters were prospectively followed up regarding immunotherapy. Of the 228 patients, 165 underwent immunotherapy using freshly prepared paternal lymphocytes and pregnancy outcome was analyzed. No mixed lymphocyte culture reaction-blocking antibodies (MLR-BAbs) were observed in these patients prior to vaccinations. Pregnancy outcome was also analyzed in such as those patients positive for MLR-BAbs and who did not undergo immunotherapy, and in patients negative for MLR-BAbs and who had become pregnant without immunotherapy.

Results
Of the 140 newly pregnant patients after immunotherapy, the pregnancy continued successfully in 110 (78.6%), and the pregnancy continued successfully in 24 of 32 patients (75.0%) who were positive for MLR-BAbs. The success rate of pregnancy was 30.0% in 18 non-immunized patients. Thus, the success rate was significantly higher among patients with immunotherapy and patients positive for MLR-BAbs than in non-immunized patients, negative for MLR-BAbs.

Conclusion
Immunotherapy using paternal lymphocytes is considered to be effective for unexplained primary recurrent aborters negative for MLR-BAbs.

Introduction

Although the etiology of recurrent spontaneous abortion, defined as three or more consecutive early pregnancy losses, is often unclear, several investigators have reported the occurrence of immunologically explainable recurrent spontaneous abortions. Immunotherapy for these patients using the husband's or a third party's leukocytes has been reported for the past quarter of a century.[1–7] The efficacy of this modality, however, is controversial, even among studies with randomized controlled trial.[8–11] This is mainly because of the selection criteria that were adopted, or of the procedure of the immunotherapy. In this study, we show the results of immunotherapy for unexplained primary recurrent aborters using freshly prepared lymphocytes from the husband and attempt to examine the efficacy of the therapy, especially in patients negative for blocking antibodies evaluated by a mixed lymphocyte culture reaction between spouses (MLR-BAbs).

Materials and methods

Patients

A total of 228 primary recurrent aborters, presenting between January 1983 and December 2005, took part in the study. All had provided informed consent. Each participant cohabiting with a single partner had experienced three or more consecutive, confirmed first trimester (i.e. before 14 weeks of gestation) spontaneous abortions. All had experienced no other pregnancy, and so were diagnosed as primary recurrent aborters. None of the participants had indication for presence of any genetic impairment, mullerian anomaly, hormonal deficiency, infectious disease, metabolic disorder, or autoimmune abnomalities, such as antiphospholipid antibodies or lupus anticoagulant disorder, in the course of our systemic work-up.

The patients were initially divided into two groups according to the presence or absence of mixed lymphocyte culture reaction – blocking antibodies (MLR-BAbs) in the sera collected at the time of or after the last abortion. Those with MLR-BAbs were excluded from the immunotherapy, and the natural course of their pregnancy was observed. Sufficient information concerning immunotherapy for recurrent abortion using paternal lymphocytes was given to the patients who were negative for MLR-BAbs, and the immunotherapy was applied only to those patients who requested it. With respect to the patients who did not desire the immune-therapy, the natural course of their pregnancy was observed. If the pregnancy resulted in repeated abortion, the immunotherapy was given at the patient's request.

Thus, the patients were ultimately divided into three groups, that is, those who underwent immunotherapy (group I), those to whom immunotherapy was not given on account of the presence of MLR-BAbs (group II), and those who did not receive immunotherapy at their own request despite being negative for MLR-BAbs (group III). All patients were offered the same degree of care during their pregnancy.

The period of following up the patients, especially those who had not got pregnant, was about 5 years. A patient was deemed to have not gotten pregnant, if the patient concerned had not become pregnant for about 5 years. Concerning the patients who enrolled in this study after January, 2002, the outcome of pregnancy was determined in December, 2006.

Vaccinations Using the Husband's Lymphocytes

The vaccination procedure has been described in detail elsewhere.[3,4,12] Lymphocytes from 100 mL of heparinized peripheral blood of the husband concerned of each patient, in the experimental group, irradiated with 30 Greys of X-rays to prevent any graft-versus-host (GVH) reaction, were suspended in approximately 1 mL of normal physiological saline solution. Each such cell suspension was i.d. injected into the corresponding patient in the experimental group I, immediately after its preparation. Once MLR-BAbs appeared in the sera following a series of vaccinations, the patients were allowed to become pregnant. In our earlier study, the MLR-BAbs were examined after each vaccination.[3] In a recent study, however, the patients underwent two vaccinations 1 month apart, and then given a third vaccination if the MLR-BAbs were not still detected.[4] If the MLR-BAbs could not be detected after the third vaccination, the patients were allowed to become pregnant, and an additional vaccination was given early in the pregnancy.

Mixed Lymphocyte Culture Reaction-Blocking Assay

The blocking effect of sera was investigated in a one-way MLR between spouses. Lymphocytes were collected from heparinized blood via Ficoll-Hypaque gradient centrifugation. Mixed culturing of mitomycin C-treated stimulator cells of the husband and responder cells of the patient was performed for 6 days in a microtiter plate in RPMI 1640 medium containing either pooled human AB serum or test serum. The cultured cells were harvested onto a glass fiber filter after 18 hr of pulsing with ^{3}H-thymidine. DNA synthesis was evaluated by liquid scintillation counting, and the blocking effect (BE) was calculated with the formula

$$\mathrm{BE} = (1 - \text{mean cpm of culture in tested serum} / \text{mean cpm of culture in AB serum}) \times 100(\%)$$

A 22% or more MLR-blocking effect was determined as significant, and designated a positive reaction for MLR-BAbs, as reported.[3,4,12]

The procedure used for immunization and the method used for testing MLR-BAbs have been

validated, as one of the authors of this paper has been directly engaged in the immunization and MLR-BAbs test throughout this study.

Statistical Analysis

A non-paired *t*-test was used to analyze whether a significant difference exists among the mean age or the mean number of spontaneous abortions in experimental groups I, II, and III. A chi-squared analysis with Yates' correction or Fisher's exact probability test was used to analyze the probability that pregnancy outcome differed among groups I, II, and III.

Results

The patients accrued in this study are shown in Fig. 1. Of 228 patients, 179 (78.5%) were found to be negative for MLR-BAbs with the MLR-blocking assay, using sera collected at the time of or just after their last abortion. Immunotherapy was given to 156 patients who were negative for MLR-BAbs, at their request. The remaining 23 patients had not opted for immunotherapy, and 18 patients experienced 20 pregnancies without immunotherapy (group III) (pregnancy rate: 78.3%). Of these cases, six pregnancies resulted in normal term delivery, and the remaining 14 pregnancies resulted in repeated spontaneous abortion (Success rate; 30.0%). Nine patients in these unsuccessful cases desired immunotherapy at a subsequent stage. Thus, immunotherapy using paternal lymphocytes was given to 165 patients (group I). Of the 49 patients who were positive for MLR-BAbs, 32 have so far experienced further pregnancy later (group II) (pregnancy rate; 65.3%).

The mean patient age and mean number of abortions did not differ significantly among these three groups (Table I).

For 25 of the 32 patients (78.1%) of group II, the pregnancy culminated in delivery. Of these 25, 21 gave birth to mature infants. One light-for-date infant (an infant whose body weight was less than the 10th percentile of the distribution of the general population (neonates) in Japan[13]) was born in the 38th week of gestation with no anomalies, and one infant was born premature in the 31st week of gestation. Three patients had infants with a major anomaly: one of these infants died just before delivery due to severe omphalocele, one was saved by surgery for intestinal atresia, the other was saved by surgery for meconium peritonitis. Pregnancy had resulted in repeated spontaneous abortion in seven cases. Thus, the success rate in this group was 75.0% (24 of 32).

As mentioned above, 165 patients had undergone vaccination with their husbands' lymphocytes. MLR-BAbs were detected after one or two vaccinations in 148 of these, and after the third vaccination in 14 more patients. Thus, the MLR-BAbs were found in 98.2% of vaccinated patients. In the remaining three patients, no MLR-BAbs were detected even after the third vaccination, and an additional vaccination was given early in the pregnancy.

Of 165 patients, 140 experienced new pregnancies (pregnancy rate; 84.8%), and 110 had their pregnancy continue successfully (success rate: 78.6%). All of these 110 patients have already experienced delivery, and 101 delivered normal mature infants in the 36th week of gestation or later. Four light-for-date infants were born at 36 weeks of gestation or later with no anomaly, and four infants were born as premature delivery in the 28th, 32nd, 33rd, and 34th week of gestation. A major fetal anomaly was observed in one infant diagnosed as having Delange syndrome, who had survived after delivery. Pregnancy resulted in repeated spontaneous abortion in the remaining 28 cases, and in ectopic pregnancy in two cases.

The outcome of pregnancy in groups I, II, and III is shown in Table II. The rate of success was significantly higher in group I and II than group III (78.6% *versus* 30.0%, $P < 0.000001$, 75.0% *versus* 30.0%, $P < 0.001$, respectively).

The outcome of pregnancy in group I according to the number of vaccination(s) necessary to test positive for MLR-BAbs is shown in Table III. The rate of successful pregnancy among the patients in whom the MLR-BAbs appeared after one or two vaccination(s) was 76.0% (96 of 125 cases). In the patients in whom the MLR-BAbs showed up after three vaccinations was 91.7% (11 of 12 cases). The rate was 100% in the group of patients in whom MLR-BAbs could not be observed after three vaccinations and an additional vaccination was applied at an early stage of pregnancy (three of three cases). The success rate did not differ significantly among these three groups.

Discussion

The outcome of immunotherapy using the husband's lymphocytes for unexplained primary recurrent

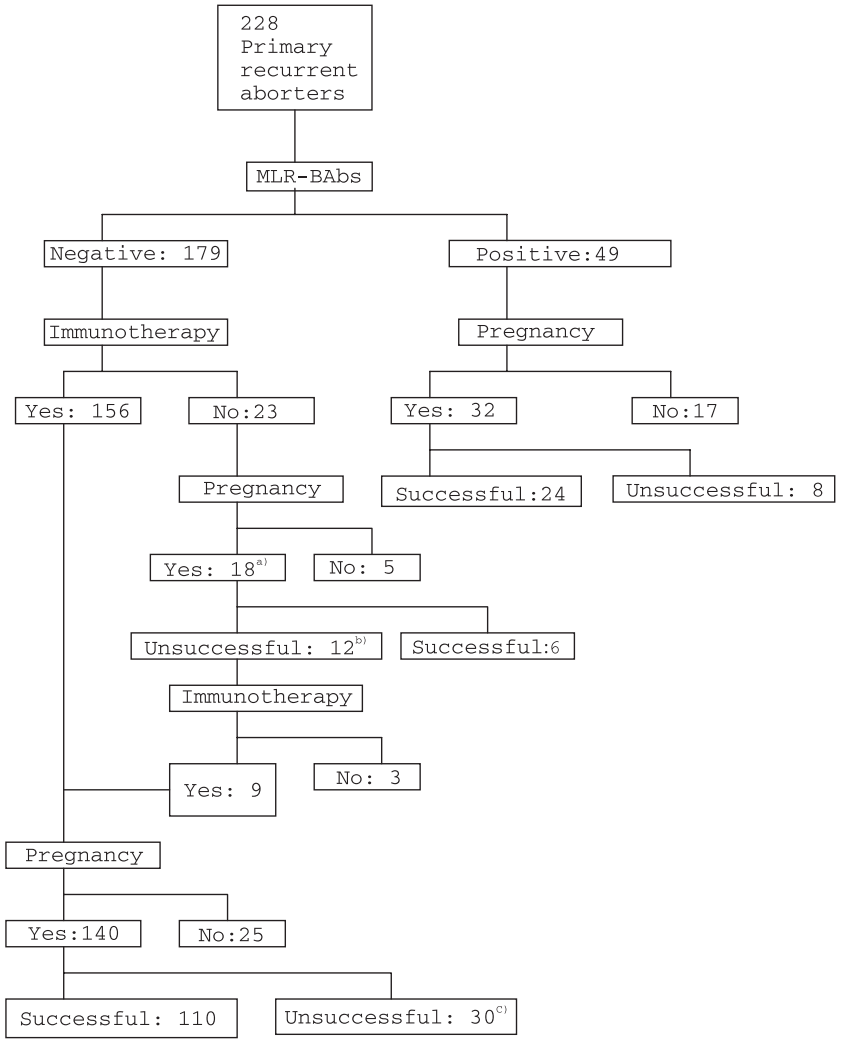

Fig. 1 Accrual of the patients. The study included cases from January 1983 to December 2005. (a) These 18 patients experienced 20 pregnancies. (b) These 12 patients experienced 14 abortions. (c) Two cases of ectopic pregnancy were included.

Table I Characteristics of Patients in Group I, Group II, and Group III

	No. of patients	Age (range)	No. of abortions
Group I	165	30.5 ± 4.41 (22–43)	3.22 ± 0.49
Group II	49	31.2 ± 4.53 (22–40)	3.24 ± 0.60
Group III	23	30.1 ± 4.98 (23–40)	3.09 ± 0.29

No significant difference was observed among three groups concerning mean age and mean number of abortions.

abortion in this series was improved compared with that of the control patients who did not undergo immunotherapy. The results of some earlier case-controlled studies of immunotherapy for recurrent spontaneous abortions show that the outcome of subsequent pregnancies is significantly improved by the injection of paternal lymphocytes as compared to the outcome after the injection of autologous cells.[8,9] A worldwide meta-analytic study has concluded that immunization may be highly effective, although for only a small number of patients.[5] Recently, Pandey et al. reported that a double-blind randomized trial of paternal lymphocyte immunization for women with recurrent spontaneous abortion revealed the significant improvement in pregnancy outcome in the patient group.[11,14] Although the current study is not a case-controlled trial, and accordingly has less influence than a randomized trial, the results are still considered to suggest the efficacy of

Table II Comparison of Pregnancy Outcome in Group I, Group II, and Group III

	Group I (n = 140)	Group II (n = 32)	Group III (n = 20)[a]
Normal delivery at week 36 of gestation or later	101	20	6
Light-for-date infant delivery at week 36 of gestation or later	4[b]	1[c]	0
Preterm delivery before week 36 of gestation	4[d]	1[e]	0
Fetus with major anomaly[f]	1	3	0
Spontaneous abortion	28	7	14
Ectopic pregnancy	2	0	0
Number and rate of successful pregnancy	110 (78.6%)[g]	28 (75.0%)[h]	6 (30.0%)[g,h]

[a]Twenty pregnancies in 18 patients.
[b]The pregnancy outcome was; 38 weeks, 1858 g; 38 weeks, 1865 g; 36 weeks, 1865 g; 36 weeks, 2044 g.
[c]The pregnancy outcome was; 38 weeks, 1816 g.
[d]The pregnancy outcome was; 28 weeks, 1088g; 32 weeks, 1766g; 33 weeks, 1524g; 34 weeks, 2118 g.
[e]The pregnancy outcome was; 31 weeks, 1714 g.
[f]The details were described in the text.
[g]Group I *versus* group III, $P < 0.000001$ by chi-squared analysis.
[h]Group II *versus* group III, $P < 0.001$ by chi-squared analysis.

Table III Comparison of Pregnancy Outcome in Group I According to the Number of Vaccination(s) Necessary for Positive MLR-BAbs

	One or two vaccinations (n = 125)	Three vaccinations (n = 12)	No appearance of MLR-BAbs and additional vaccination at the early stage of pregnancy (n = 3)
Normal delivery at week 36 of gestation or later	87	11	3
Light-for-date infant delivery at week 36 of gestation or later	4[a]	0	0
Preterm delivery before week 36 of gestation	4[a]	0	0
Fetus with major anomaly	1[b]	0	0
Spontaneous abortion	27	1	0
Ectopic pregnancy	2	0	0
Number and rate of Successful pregnancy	96 (76.8%)[c]	11 (91.7%)[c]	100 (100%)[c]

MLR-BAbs, mixed lymphocyte culture reaction-blocking antibodies.
[a]The details were described in Table II.
[b]The details were described in the text.
[c]No significant difference was observed among the three groups.

immunotherapy for unexplained primary recurrent spontaneous abortion.

As one of the selection criteria for patients to be considered eligible for immunotherapy, the presence or absence of MLR-BAbs before immunotherapy was considered to be important. This has been emphasized by several investigators, including ourselves.[3,4,6,11,14–16] Park et al. reported that the blocking effect index (BEI), calculated using a modification of our MLR blocking assay, was a reliable indicator of the outcome of subsequent pregnancies in unexplained recurrent aborters following immunotherapy.[16]

As to the origin of naturally acquired MLR-BAbs, there is a possibility that stimulation by paternally derived antigens on the trophoblasts of previous conceptions or by the exposure of semen might generate the MLR-BAbs.

The MLR-BAbs were used in a recent double-blind randomized trial by Pandy et al. as selection criteria for immunized patients.[11] The results obtained in the present study show that the patients negative for MLR-BAbs benefit from immunotherapy with the husband's lymphocytes. In patients with MLR-BAbs, the success rate of pregnancy was significantly higher than that in the control group, while the rate

was not significantly different from that in the treated patients. Thus, it can be concluded from this result that immunotherapy was not of additional value for the patients who were positive for MLR-BAbs.

As a negative report concerning the immunotherapy, Porter et al. found that immunotherapy for unexplained recurrent aborters has no beneficial effect, upon analyzing the Chochrane database.[17] The conclusion was critically influenced by the negative results of a randomized trial by Ober et al.[10] As has been pointed out by Clark et al.[18], this trial unfortunately showed considerable flaws in its design. Moreover, the discrepancy between their findings and ours is probably due to the fact that they did not use selection criteria, such as MLR-BAbs, for the recipients of immunotherapy, and it is possible that the immunotherapy was given to patients who did not need it. Another explanation for the discrepancy is that they used lymphocytes stored overnight before the vaccination and we used freshly prepared lymphocytes. Recently, Clark et al. reported that transfusion-related immunomodulation can enhance the growth and survival of the feto-placental unit via CD200, and transfusion-related immunomodulation was lost if the transfused cells were stored overnight.[19,20]

For two decades, the so-called immunotrophic theory, whereby some cytokines produced by maternal cells which recognize fetal antigens, promote the proliferation of trophoblastic cells and sustain a pregnancy, has been thought to be significant for the immunological maintenance of pregnancy.[21] Moreover, some investigators demonstrated the importance of a T-helper 2 (Th2) bias for normal pregnancy, indicating the crucial role of the activation of maternal humoral immunity following recognition of fetal antigens during pregnancy.[22–25] Although the theory that the maternal Th2/Th1 dichotomy plays an important role has been recently challenged by several researchers,[26,27] some studies demonstrated that a predominance of Th2 over Th1 was induced in patients by immunization with the husband's lymphocytes, which was correlated with the efficacy of the therapy.[28,29]

In this study, the appearance of MLR-BAbs was almost ubiquitous (98.2% of vaccinated patients), and the success rate of pregnancy did not differ significantly with the number of vaccination(s) necessary for MLR-BAbs to appear. Such a ubiquitous appearance of MLR-BAbs after immunotherapy may indicate the generation of an appropriate immune reaction in patients, i.e., the induction of a predominance of humoral immunity, which is considered to contribute to a successful continuation of the subsequent pregnancy.

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