Alteration of TH1 and TH2 cells by intracellular cytokine detection in patients with unexplained recurrent abortion before and after immunotherapy with the husband's mononuclear cells

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Objective: To elucidate the possible mechanisms of immunotherapy for unexplained recurrent aborters using their husband's mononuclear cells.
Design: Prospective clinical study.
Setting: Institutional practice at the Outpatient Clinic for Infertility, Niigata University Medical Hospital.
Patient(s): Fifty-two unexplained recurrent aborters were chosen as an experimental group.
Intervention(s): Each patient was injected with her husband's mononuclear cells as immunotherapy. Peripheral blood was obtained from the patients.
Main Outcome Measure(s): The percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio were analyzed in the patients before and after immunotherapy. The same analyses were performed in the successful and the unsuccessful group.
Result(s): To date, 42 of the 52 patients have become newly pregnant. Of the 42, 34 patients have already delivered (successful group) and 3 are now pregnant, while the remaining 8 cases experienced repeated abortion (unsuccessful group). The percentage of TH2 cells significantly increased in the total patient population, while the TH1/TH2 ratio significantly decreased in the total patient population and in the successful group.
Conclusion(s): These findings suggest that immunotherapy with the husband's mononuclear cells for unexplained recurrent abortion induces a dominant state of TH2 cells in the patients. (Fertil Steril® 2006;85:1452–8. ©2006 by American Society for Reproductive Medicine.)

Key Words: Unexplained recurrent abortion, TH1, TH2, immunotherapy, mechanisms

The observation that the human fetus or feto-placental unit is a semiallograft and thus antigenically foreign to the mother, yet does not undergo maternal immune rejection in normal pregnancy, was first made just a half century ago (1). However, successful human pregnancy remains an immunologic enigma, and our understanding of its pathologic manifestations is limited.

It has recently been found that the production of a diversity of cytokines by maternal immune-competent cells in the decidual tissues promotes the growth of trophoblastic cells, and a shift to TH2-related humoral immunity from TH1-driven, cell-mediated immunity was suggested to be beneficial for immunologically successful continuation of pregnancy (2–9).

On the contrary, the lack of such an appropriate immune reaction is considered to cause recurrent spontaneous abortion, especially unexplained recurrent spontaneous abortion (10, 11).

Some investigators, including ourselves, demonstrated the possible efficacy of immunotherapy for unexplained recurrent aborters using paternal mononuclear cells (12–19).

The immunotherapy mechanisms have not yet been fully elucidated, but it is possible that immunotherapy will give rise to a TH1/TH2 balance in patients with unexplained recurrent abortion who undergo immunotherapy.

To date, however, the association between immunotherapy and the TH1/TH2 balance has not yet been fully elucidated. In this context, we analyzed the alteration in the TH1/TH2 balance in a patient population of unexplained

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recurrent aborters who underwent immunotherapy to elucidate the possible immunotherapy mechanisms.

MATERIALS AND METHODS

Fifty-two unexplained recurrent aborters who had sustained three or more consecutive first-trimester spontaneous abortions were chosen as an experimental group. The age of the patients ranged from 23 to 45 with a mean of 32.0 years old.

All of them had experienced three or more consecutive confirmed first trimester spontaneous abortions with one partner. None of the participants had any genetic impairment, Mullerian anomaly, hormonal deficiency, infectious disease, metabolic disorder, or autoimmune abnormalities, such as positive antiphospholipid antibodies or lupus anticoagulant, in our systemic work-up.

In addition, tests for thrombophilic status, such as protein C activity, protein S activity, and thrombin-anti-thrombin III complex, were routinely performed for all patients.

All patients were healthy except for their history of recurrent abortions, and were negative for blocking antibodies, identified by a one-way mixed lymphocyte culture reaction (responder: patient; stimulator: husband), in their sera. Each patient was injected with her husband's mononuclear cells with informed consent.

The percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio was compared before and after immunotherapy in the total patient populations, in the patients whose pregnancies continued successfully after immunotherapy (successful group), and in the patients whose pregnancies had resulted in repeated abortion (unsuccessful group). Institutional Review Board approval was obtained before the studies.

Immunotherapy Procedure Using the Husband's Mononuclear Cells

Details of the immunotherapy procedure were described elsewhere (15, 16, 18, 20). Mononuclear cells from about 100 mL of heparinized peripheral blood of the husband, irradiated with 30 Grey of X-rays to prevent any graft-versus-host (GVH) reaction, were suspended in approximately 1 mL of normal physiological saline solution. This cell suspension was intradermally injected into the patients after obtaining informed consent. After the appearance of mixed lymphocyte culture reaction-blocking antibodies (MLR-BAbs) in patients' sera following a series of two or more injections 1 month apart, the patients were allowed to become pregnant.

To date, a significant level of MLR-BAbs has been detected after a series of the injections in all patients. Analyses of CD4 positive cells, TH1 cells, and TH2 cells were performed just after the last abortion as preimmunotherapy tests, and were also performed about 4 weeks after the last injection as postimmunotherapy tests.

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 in a microtiter plate in Roswell Park Memorial Institute (RPMI) 1640 containing either pooled human AB (blood type of AB) serum or tested serum for 6 days. The cultured cells were harvested onto a glass fiber filter after a pulse time of 18 hours with ^{3}H-thymidine. The DNA synthesis was evaluated by liquid scintillation counting, and the blocking effect (BE) was calculated by the formula:

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

The significant level of the MLR-blocking effect was determined to be more than 22%, which was designated as positive for MLR-BAbs, as previously reported (15, 16, 18, 20).

Analyses of CD4-Positive Cells

One hundred microliters of whole blood collected from patients were incubated with 10 μL of appropriately titered fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (NU-TH/I-FITC, Nichirei, Japan) in an ice bath for 30 minutes, then treated with 2 mL of a lysing agent (0.83% ammonium chloride) for 10 minutes at room temperature.

The pellet was washed once in phosphate-buffered saline (PBS), and the cells were then diluted to a final volume of 2 mL in PBS. The antibody-reacted cells were analyzed with a Flowcytometer (Ortho Clinical Diagnostics, Raritan, NJ).

Analyses of the TH1 and TH2 Cells

Cells with TH1 and TH2 were determined by detecting the intracellular interferon (IFN)-gamma and IL-4 production (21–23).

Peripheral heparinized venous blood cells were washed three times in Hanks' balanced salt solution and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 50 U/mL of penicillin, and 50 μg/mL of streptomycin. After 2 hours' cultivation in a culture dish, nonadherent cells were collected and stimulated with 25 ng/mL of phorbol-12-myristate-13-acetate and 1 μmol/l of ionomysin in the presence of 10 μg/mL of brefeldin A (Sigma, St. Louis, MO) for 4 hours at 37°C with 7% CO_2 in RPMI 1640 supplemented with FCS.

Peridininchlorophyll protein (PerCP)-conjugated anti-CD4 and PerCP-conjugated antimouse immunoglobulin G1 (IgG1) were used to analyze cell surface antigens. The FITC-conjugated IFN-gamma and phycoerythrin (PE)-conjugated anti-IL-4 (Becton Dickinson Immunocytometry Systems [BDIS], Mountain View, CA) were used to analyze

intracellular cytokines. The FITC-conjugated IgG2a and PE-conjugated IgG1 antibodies were used as control antibodies.

An anti-CD4-PerCP antibody was added to the lymphocytes and they were incubated for 15 minutes at room temperature. Then, the cells were washed with PBS with 0.1% bovine serum albumin. The cell pellet was fixed with lysing solution (BDIS) and permeability was achieved with a permeabilizing solution (BDIS) according to the manufacturer's instructions. Anti-IFN-gamma FITC and anti-IL-4 PE were added, and incubation was performed for 30 minutes at room temperature. For control samples, FITC-conjugated IgG2a and PE-conjugated IgG1 antibodies were used in the same reaction.

The samples were analyzed on a FACScan (BDIS) using Cell Quest Software (BDIS). Dead cells and monocytes were excluded from lymphocytes initially by side scatter gating and then by forward scatter gating. Cell populations were defined as follows: TH1: IFN-gamma-positive and IL-4-negative; TH2: IFN-gamma negative and IL-4 positive.

STATISTICAL ANALYSES

A paired *t*-test was used to analyze the significance of the difference in the percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio before and after immunotherapy.

RESULTS

The mean percentage of CD4-positive cells in the total patient population with unexplained recurrent abortion before immunotherapy was 44.6% ± 7.29%, and the mean percentage after immunotherapy was 43.7% ± 7.70%. The mean percentage of TH1 cells in all patients with unexplained recurrent abortion before immunotherapy was 21.4% ± 7.88%, and the mean percentage after immunotherapy was 21.0% ± 5.97%.

Thus, the percentages of CD4-positive cells and TH1 cells were not significantly different before and after immunotherapy in this population.

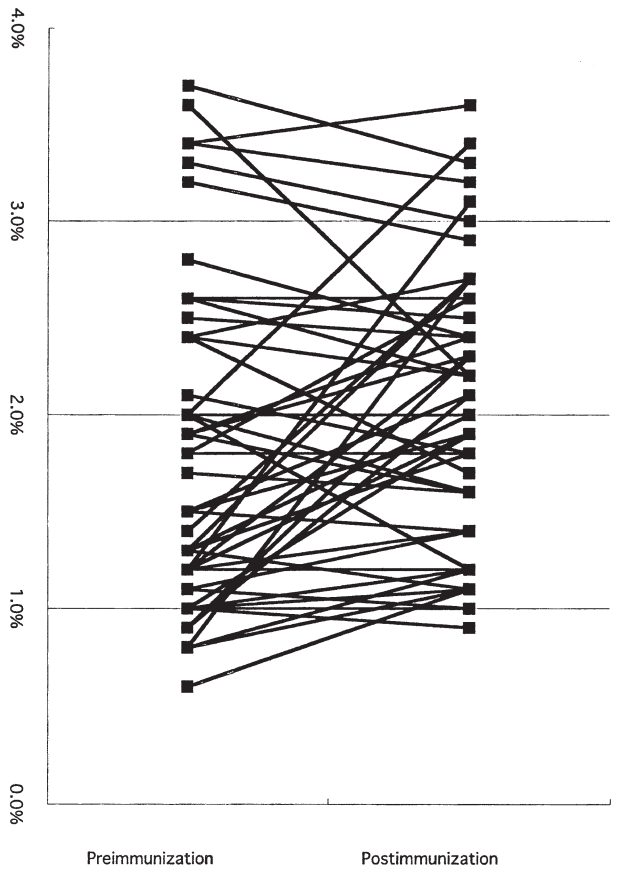
The mean percentage of TH2 cells in patients with unexplained recurrent abortion before immunotherapy was 1.78% ± 0.82%, and the mean percentage after immunotherapy was 2.03% ± 0.71% (Fig. 1). Thus, the percentage of TH2 cells significantly increased after immunotherapy compared with that before immunotherapy (P=.012, paired *t*-test).

The mean of TH1/TH2 ratio in patients before immunotherapy was 14.0 ± 7.21, and that after immunotherapy was 11.7 ± 5.26 (Fig. 2); therefore, the mean TH1/TH2 ratio significantly decreased after immunotherapy compared with that before immunotherapy (P=.002, paired *t*-test).

To date, 42 of the 52 patients have become newly pregnant. Of the 42, the pregnancy continued in 34 patients (81.0%), while the remaining 8 cases experienced repeated abortion (unsuccessful group). Of the 34 patients in whom

FIGURE 1

The change in the mean percentage of TH2 cells in the total patient population before immunotherapy (1.78% ± 0.82%) and after immunotherapy (2.03% ± 0.71%). The percentage of TH2 cells significantly increased after immunotherapy compared with that before immunotherapy (P=.012, paired *t*-test).



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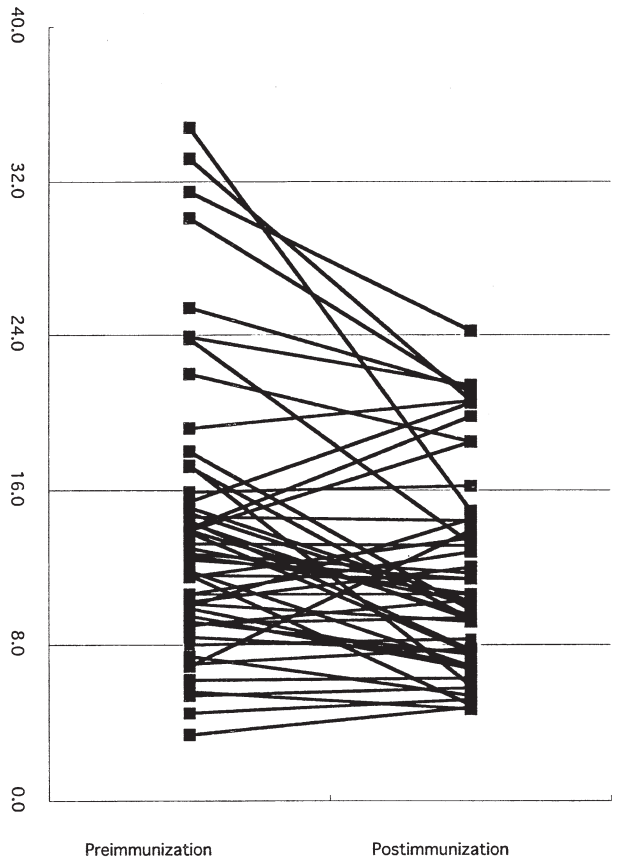
the pregnancy continued, 31 have already delivered babies, and the remaining 3 are now pregnant (their gestational weeks [Gw] are 29, 22, and 17, respectively).

Twenty-eight patients of 31 experienced term delivery, and 3 experienced preterm delivery (1 of them was singleton delivery at the 36th Gw, 1 was a twin delivery at the 36th Gw, and the remaining 1 was a singleton delivery at the 34th Gw). All infants born to the patients displayed an uneventful neonatal course. We named the patients who had already experienced delivery the "successful group" (n = 31).

The mean percentage of CD4-positive cells in the successful group before immunotherapy was 42.6% ± 6.13%, and the mean percentage after immunotherapy was 42.6% ± 7.00%, whereas the mean percentage of TH1 cells in the successful group before immunotherapy was 23.1% ± 7.70%, and the mean percentage after immunotherapy was 22.1% ± 5.15%.

FIGURE 2

The change in the TH1/TH2 ratio in the total patient population before immunotherapy (14.0 ± 7.21) and after immunotherapy (11.7 ± 5.26). The mean TH1/TH2 ratio significantly decreased after immunotherapy compared with that before the immunotherapy (P=.002, paired t-test).



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The mean percentage of TH2 cells in the successful group before immunotherapy was 1.81 ± 0.75%, and the mean percentage of TH2 cells after immunotherapy was 2.01 ± 0.75% (Fig. 3). Thus, the percentages of CD4 positive cells, TH1 cells, and TH2 cells were not significantly different between before and after immunotherapy in the successful group.

The mean TH1/TH2 ratio in the successful group before immunotherapy was 14.5 ± 6.74, and the mean TH1/TH2 ratio after immunotherapy was 12.6 ± 5.57 (Fig. 4). Thus, the mean TH1/TH2 ratio significantly decreased after immunotherapy compared with that before immunotherapy (P =.018, paired t-test).

The mean percentage of CD4-positive cells in the unsuccessful group before immunotherapy was 46.7 ± 7.11%, and the mean percentage of CD4-positive cells after immunotherapy was 44.0 ± 5.34%. The mean percentage of TH1 cells in the unsuccessful group before immunotherapy was 17.5 ± 7.24%, and the mean percentage of TH1 cells after immunotherapy was 18.4 ± 7.82%.

The mean percentage of TH2 cells in the unsuccessful group before immunotherapy was 1.46 ± 0.99%, and the mean percentage of TH2 cells after immunotherapy was 1.76 ± 0.82%. The mean TH1/TH2 ratio in the unsuccessful group before immunotherapy was 16.0 ± 11.0, and the mean TH1/TH2 ratio after immunotherapy was 11.5 ± 4.9 (Fig. 5).

The percentages of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio were not significantly different between before and after immunotherapy in the unsuccessful group (paired t-test).

DISCUSSION

In this study, we analyzed the changes in the percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2

FIGURE 3

The change in the percentage of TH2 cells in successful group before immunotherapy (1.81% ± 0.75%) and after immunotherapy (2.01% ± 0.75%). No significant difference was observed between before and after immunotherapy.

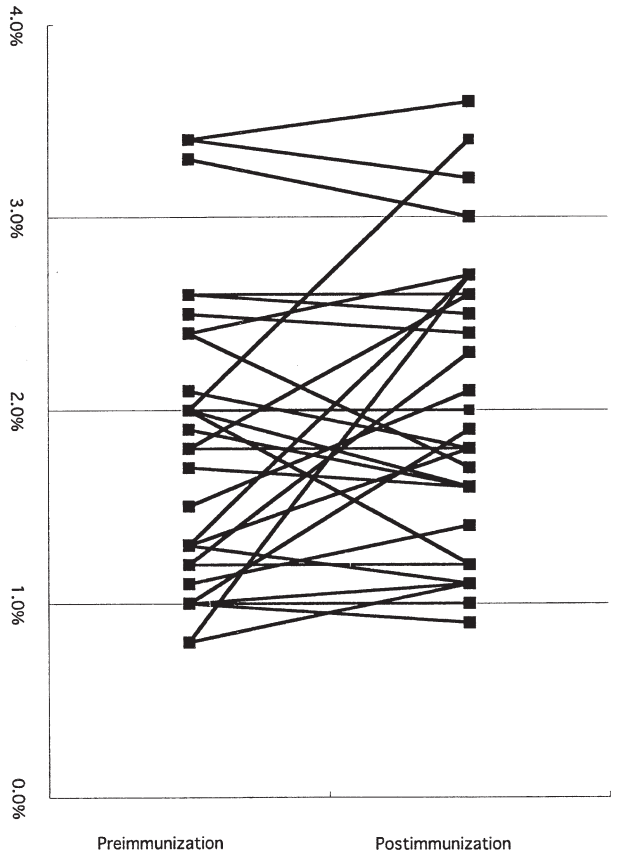

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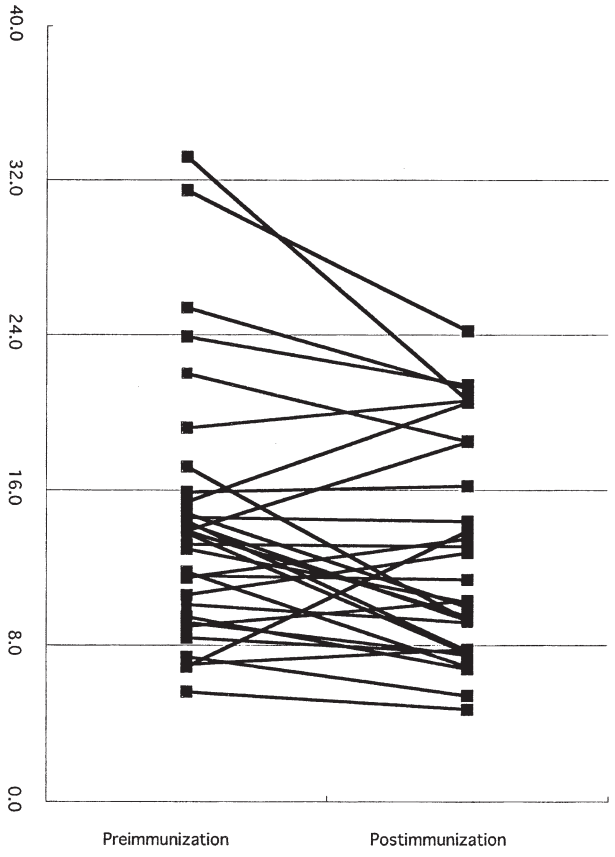
FIGURE 4

The change in the TH1/TH2 ratio in successful group before immunotherapy (14.5 ± 6.74) and after immunotherapy (12.6 ± 5.57). The mean TH1/TH2 ratio in the successful group significantly decreased after immunotherapy compared with that before immunotherapy (P=.018, paired t-test).



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ratio in patients with unexplained recurrent abortions before and after immunotherapy with the husband's mononuclear cells. The changes were also analyzed in a successfully immunized group and an unsuccessful group.

It was observed that the percentage of TH2 cells significantly increased with immunotherapy in the total patient population, and that the TH1/TH2 ratio significantly decreased with immunotherapy in the total patient population and in the successful group, although no significant change was observed in the unsuccessful group.

As antigens expressed on the surface of fetal or placental tissues possibly induce the alloimmune response of the mother, there appear to be certain immunologic mechanisms that sustain the continuation of normal pregnancy.

Progress in understanding the immunologic mechanisms for continuation of pregnancy has been made in studies of women with unexplained recurrent abortion over the past three decades. That is, several investigators have reported the existence of immunologically explainable recurrent spontaneous aborters, and immunotherapy for these patients using their husbands' or a third party's leukocytes has been reported by several authors, including ourselves (12–19).

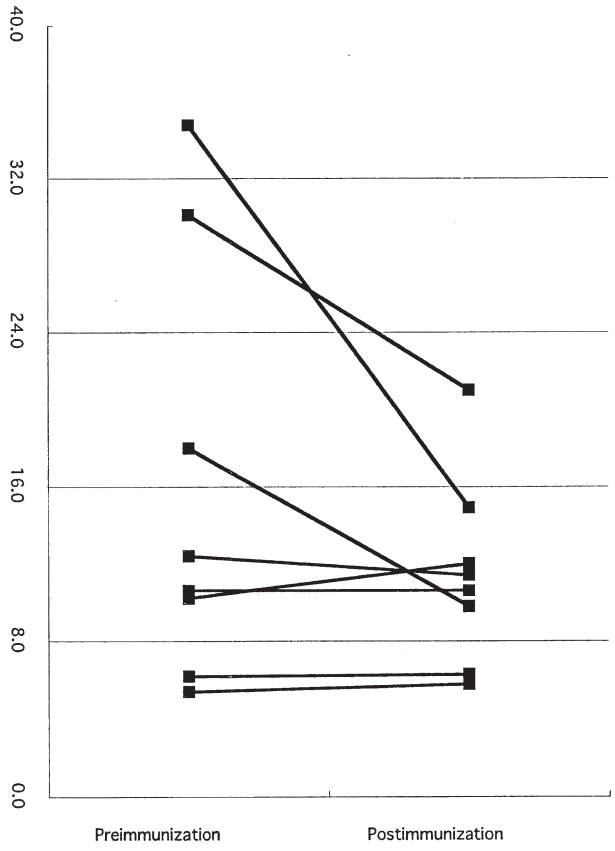
The results of some case-controlled immunotherapy studies on recurrent spontaneous abortions indicate that the outcome of subsequent pregnancies is significantly improved by injection of paternal lymphocytes, as compared with that after injection of autologous cells (12, 13, 19), although Ober et al. reported the ineffectiveness of this treatment (24).

A worldwide meta-analysis study has concluded that immunization may be highly effective, although only for a small number of patients who have the indication (25).

Although immunotherapy is considered possibly efficacious, the underlying mechanisms have not yet been fully

FIGURE 5

The change in the TH1/TH2 ratio in the unsuccessful group before immunotherapy (16.0 ± 11.0) and that after immunotherapy (11.5 ± 4.9). No significant difference was observed between before and after immunotherapy.



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elucidated. Several investigators reported the production of blocking antibodies in a patient's serum, which inhibited the response of autologous lymphocytes to transfused paternal lymphocytes (14, 17, 19).

We reported that patients with negative MLR-BAbs benefited from immunotherapy with the husband's lymphocytes in both unexplained primary recurrent abortions (15) and secondary recurrent abortions (16), and also that significant MLR-BAbs were induced in almost all patients who underwent immunotherapy with their husband's lymphocytes (15, 16, 18, 20).

Concerning the changes in cellular immunity before and after immunotherapy, we previously reported that a significant decrease in the CD4/CD8 ratio was observed in immunized patients mainly because of an increase in the CD8 subpopulation (26).

In this study, we analyzed the alterations in the CD4 cell population, and no significant change was observed in immunized patients, which is the same tendency as the previous report. On the other hand, Miki et al. reported that immunotherapy had no influence on natural killer receptor status (27).

As one of the important mechanisms which immunologically sustain pregnancy, Wegmann et al. proposed immunotrophic theory (2, 3), whereby some cytokines produced by maternal cells, which recognize fetal antigens, promote the proliferation of trophoblastic cells and sustain pregnancy continuation.

Following this theory, the "TH1/TH2 paradigms" theory was proposed. TH1 and TH2 cells are the major subsets of fully differentiated CD4-positive T cells, and their distinctive functions in immune responses correlate with their distinctive cytokine secretion patterns (28).

In this theory, a TH2 cell bias against TH1 cells is important for normal pregnancy, indicating the crucial role of the activation of maternal humoral immunity following recognition of fetal antigens during pregnancy (4–9).

Regarding the TH1/TH2 balance in patients with recurrent abortion, Hill first reported increased TH1 cytokine production by peripheral lymphocytes exposed to JEG3 choriocarcinoma stimulation (10). Lim et al. reported that levels of TH1 cytokines were significantly higher in women with recurrent abortions compared with normal controls (11).

In this context, it is possible that immunotherapy affects the TH1 cell and TH2 cell balance in immunized patients. Hayakawa et al. reported that a significant decrease in TH1 cells, a significant increase in TH2 cells, and a significant decrease in the TH1/TH2 ratio was observed in 12 patients with recurrent abortion after immunotherapy (29).

In this study, we increased the number of patients with unexplained recurrent abortion and analyzed the alterations in CD 4-positive cells, TH1 cells, and TH2 cells.

A significant increase in TH2 cells and a significant decrease in the TH1/TH2 ratio were observed in the total immunized population, although no significant change could be observed concerning TH1 cells.

Moreover, we analyzed the alterations in TH1 cells, TH2 cells, and the TH1/TH2 ratio both in a successful group and an unsuccessful group; a significant decrease in the TH1/TH2 ratio was observed in the successful group, while no significant change was observed in the unsuccessful group.

These data suggest that immunotherapy induces a predominant state of TH2 cells against TH1 cells, and that the induction of this predominant state might be correlated with the successful continuation of pregnancy in patients with unexplained recurrent abortion who undergo immunotherapy with their husband's lymphocytes.

The number of patients in the unsuccessful group, however, was low ($n = 8$), and it has not yet been confirmed whether the TH1/TH2 ratio was significantly changed in this group.

Moreover, pregnancy resulted in repeated abortion even in patients with a decreased TH1/TH2 ratio (Fig. 5), and the pregnancy successfully continued even in the patients with an increased TH1/TH2 ratio (Fig. 4).

The reason for this discrepancy is thought to be that the immunologically successful continuation of pregnancy cannot be explained only by the TH1/TH2 paradigm theory.

Further investigation with a larger patient population, as well as the studies to confirm the efficacy of immunotherapy, is considered crucial to reach a definite conclusion.

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