tion (MLR)-blocking assay, in the immunological successful continuation of pregnancy has been considered, as MLR-BAbs (mixed lymphocyte culture reactions) are humoral factors which control maternal immune response against paternal antigens[13,14]. We have previously reported that the titer of MLR-BAbs in multiparous women was significantly higher compared with nulliparous women or those with unexplained recurrent abortions[15], and also reported that the MLR-BAbs increased significantly during the course of a normal pregnancy[16]. Moreover, some investigators, including ourselves, have demonstrated the efficacy of immunotherapy for unexplained recurrent aborters using paternal mononuclear cells, especially for those in whom the MLR-BAbs are not detected[15,17,18,19,20]. Thus, it is possible that the MLR-BAbs reflect the TH1/TH2 balance in patients with unexplained recurrent abortion. To date, however, the association between MLR-BAbs and the TH1/TH2 balance has not yet been fully elucidated. In this context, we analyzed the correlation between the MLR-BAbs and TH1/TH2 balance in a patient population of unexplained recurrent aborters.

MATERIALS AND METHODS

Patients

Forty-five unexplained recurrent aborters who sustained three or more consecutive first-trimester spontaneous abortions with the same partner were chosen as a study population. None of the patients indicated abnormalities in our systemic analyses, such as chromosomal abnormalities, Muellerian anormalies, hormonal deficiencies, infectious diseases, metabolic disorders, or autoimmune abnormalities such as SLE or positive antiphospholipid antibodies. MLR-BAbs evaluated by MLR-blocking assay were examined in all cases, and the patients were divided into two groups: an MLR-BAbs- positive group and an MLR-BAbs-negative group. The percentage of T-helper cells (CD4 positive cells) as well as the subpopulation of T-helper cells, namely, type I helper (TH1)-cells and type II helper (TH2)-cells, were also analyzed by intracellular cytokine detection in all patients. These examinations were performed during the non-pregnant state of the patients. The mean percentage of CD4-cells, TH1-cells, TH2-cells, and the TH1/TH2 ratio were compared between the two groups. The correlation between the percentage of each cell population and MLR-blocking effect (BE), and that between the TH1/TH2 ratio and MLR-BE were analyzed. The study was approved by our institutional review board, and all examinations were performed after informed consent was obtained from the patients.

Mixed lymphocyte culture reaction-blocking assay

The blocking effect of sera was investigated in 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 on a microtiter plate in RPMI 1640 containing either pooled human AB serum or tested serum. The cultured cells were harvested onto a glass fiber filter after a pulse time of 18 h with ^{3}H-thymidine. DNA synthesis was evaluated by liquid scintillation counting, and the blocking effect (BE) was calculated by the following formula:

BE = (1-mean cpm in tested serum / mean cpm in control serum) $\times 100$ (%)

As previously reported, the significant level of BE was determined to be more than 22%, which is considered to be positive for MLR-blocking antibodies (MLR-BAbs)[15,17]. A negative MLR-BE titer was considered to be 0% when analyzing the correlation between the MLR-BE and each cell population or TH1/TH2 ratio.

Analyses of T-helper cells

One hundred microliters of whole blood collected from patients was incubated with 10 μl of an appropriately titered fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (NU-TH/I-FITC, Nichirei, Japan) in an ice bath for 30 min, and then treated with 2 ml of lysing agent (0.83% ammonium chloride) for 10 min at room temperature. The pellet was washed once in PBS (phosphate-buffered saline), 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 Diagnositcs, USA).

Analyses of the subpopulation of T-helper cells

Cells with TH1 and TH2 were determined by detecting the intracellular IFN-gamma and IL-4 production[22,23,24]. Peripheral heparinized venous blood cells were washed 3 times in Hank's balanced salt solution and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin. After 2 hours' cultivation in a culture dish, non-adherent cells were collected and stimulated with 25 ng/ml of phorbol-12-myristate-13-

Table 1. Mean age and the number of abortions in patients positive for MLR-BE and those negative for MLR-BE

	Mean age	Number of abortions
Patients positive for MLR-BE (n=12)	31.3±4.14	3.25±0.45
Patients negative for MLR-BE (n=33)	31.9±3.80	3.24±0.44

No significant differences were observed as to mean age and the number of abortions.

Table 2. Percentages of CD4-positive cells, TH1, TH2 and TH1/TH2 ratio in patients positive for MLR-BE and those negative for MLR-BE

	CD4 (%)	TH1 (%)	TH2 (%)	TH1/TH2 ratio
Patients positive for MLR-BE (n=12)	45.6 ± 6.1[a)]	17.2 ± 6.20[b)]	2.06 ± 1.11[c)]	9.87 ± 4.26[d)]
Patients negative for MLR-BE (n=33)	45.5 ± 6.6[a)]	24.1 ± 7.99[b)]	1.75 ± 0.79[c)]	17.58 ± 12.91[d)]

a) not significant; b) $p<0.01$ by Student's t-test; c) not significant; d) $p<0.005$ by Welch's t-test.

acetate and 1 μmol/l of ionomysin in the presence of 10 μg/ml of brefeldin A (Sigma, USA) 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 IgG1 (immunoglobulin G1) were used to analyze cell surface antigens. FITC-conjugated IFN-gamma and phycoerythrin (PE)-conjugated anti-IL-4 (Becton Dickinson Immunocytometry Systems, BDIS, USA) were used to analyze intracellular cytokines. FITC-conjugated IgG2a and PE-conjugated IgG1 antibodies were used as control antibodies.

An anti-CD4-PerCP antibody was added to the lymphocytes and incubated for 15 min at room temperature, and the cells were washed with PBS with 0.1% bovine serum albumin. The cell pellet was fixed with lysing solution (BDIS) and permeability established with a permeabilizing solutionnite (BDIS) according to the manufacturer's instruction. Anti-IFN-gamma FITC and anti-IL-4 PE were added and incubated for 30 min at room temperature, and 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. 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, and TH2: IFN-gamma negative and IL-4 positive.

Statistical analyses

A non-paired t-test was used to analyze the significance of the difference in the mean age and mean number of abortions between the MLR-BAbs-positive and -negative groups. A non-paired t-test was also used to analyze the significance of the difference in the percentage of CD4-cells, TH1-cells, TH2-cells, and the TH1/TH2 ratio between the two groups. The correlations between the percentages of each cell population or TH1/TH2 ratio and the MLR-BE were evaluated by linear regression analysis.

RESULTS

Positivity of MLR-blocking antibodies

Of 45 patients, 12 (26.7%) were positive for MLR-BAbs (MLR-BAbs-positive group) and 33 (73.3%) were negative for MLR-BAbs (MLR-BAbs-negative group). The mean age and the mean number of abortions did not significantly differ between the two groups (Table 1).

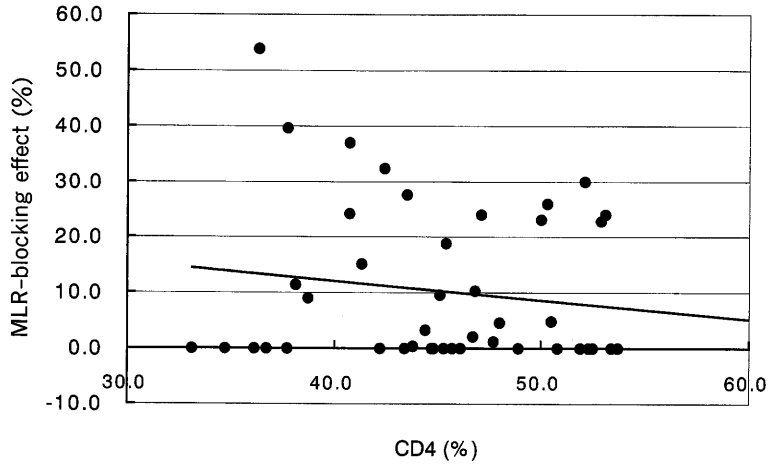

Fig. 1. Correlation between the MLR-blocking effect and the percentage of CD4-positive cells in 45 patients with unexplained recurrent abortion. No significant correlation was observed ($r = -0.158$, $P = 0.299$).

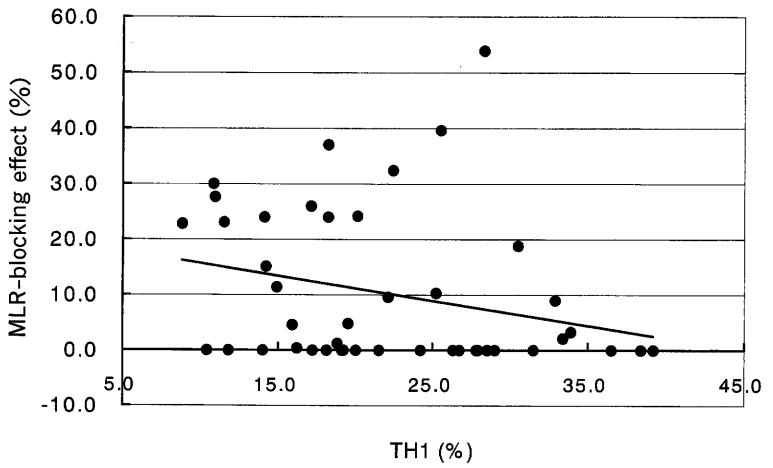

Fig. 2. Correlation between the MLR-blocking effect and the percentage of TH1-cells in 45 patients with unexplained recurrent abortion. No significant correlation was observed ($r = -0.263$, $P = 0.081$).

Percentage of CD4-cells, TH1-cells, TH2-cells, and TH1/TH2 ratio

The mean percentages of CD4-cells, TH1-cells, TH2-cells and TH1/TH2 ratio in two groups are shown in Table 2. The mean percentage of CD4-positive cells was not significantly different between the two groups, nor was the percentage of TH2-cells. The percentage of TH1-cells in the MLR-BAbs-positive group was 17.2% ± 6.20%, while that in MLR-BAbs-negative group was 24.1 ± 7.99%; thus, the percentage was significantly higher in the MLR-BAbs-negative patient group compared with the MLR-BAbs-positive patient group ($P < 0.01$, Student's t-test). The TH1/TH2 ratio was also significantly higher in the MLR-BAbs-negative patient group compared with the MLR-BAbs-positive patient group (17.6 ± 12.9% vs 9.87 ± 4.26%, $P < 0.005$ by Welch's t-test).

The correlations between the percentages of each cell population or TH1/TH2 ratio and MLR-BE

No significant correlation was observed between the percentage of CD4 positive cells and MLR-BE (Fig. 1,

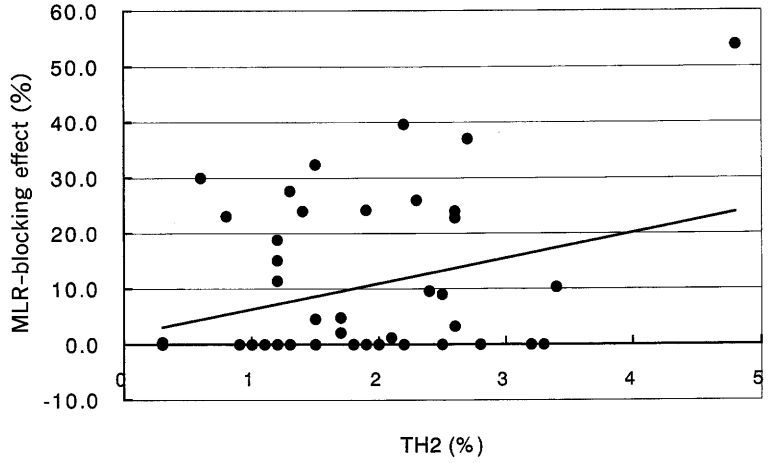

Fig. 3. Correlation between the MLR-blocking effect and the percentage of TH2-cells in 45 patients with unexplained recurrent abortion. No significant correlation was observed ($r=0.293$, $P=0.051$).

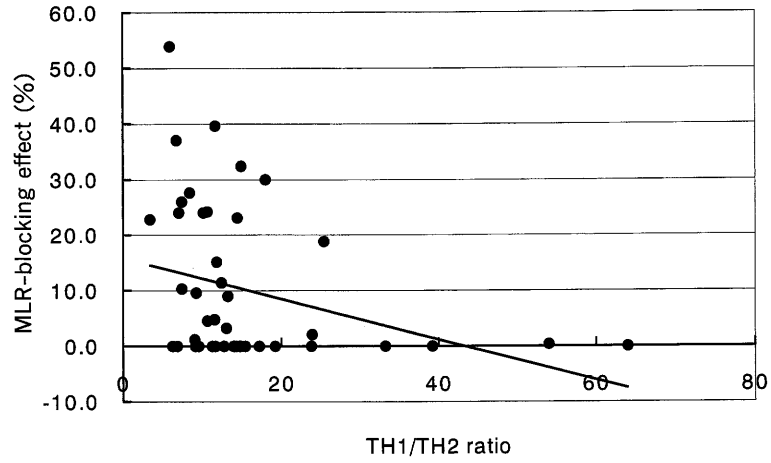

Fig. 4. Correlatron between the MLR-blocking effect and the percentage of TH1/TH2 ratio in 45 patients with unexplained recurrent abortion. Evaluation by regression analysis showed a significant negative correlation ($r=-0.308$, $P<0.05$).

$r=-0.158$, $P=0.299$). There was no significant correlation between the percentage of TH1-cells and MLR-BE (Fig. 2, $r=-0.263$, $P=0.081$), or between the percentage of TH2-cells and MLR-BE. (Fig. 3, $r=0.293$, $P=0.051$). On the other hand, MLR-BE showed a significant negative correlation with the TH1/TH2 ratio (Fig. 4, $r=-0.308$, $P<0.05$).

DISCUSSION

In this study, we analyzed the correlation among MLR-BAbs, which were evaluated by a mixed lymphocyte culture reaction between spouses and CD4-cells, TH1-cells, or TH2-cells in patients with unexplained recurrent abortions. It was suggested that the MLR-BAbs reflect the TH1/TH2 balance in patients with unexplained recurrent abortion.

As antigens expressed on the surface of fetal or

placental tissues possibly induce the allo-immune response of the mother, there appear to be certain immunologic mechanisms which sustain the continuation of a normal pregnancy. As one of these important mechanisms, Wegmann et al. proposed an immunotrophic theory[2,3], whereby some cytokines produced by maternal cells which recognize fetal antigens promote the proliferation of trophoblastic cells and ensure the continuation of the pregnancy. Following this theory, the "TH1/TH2 paradigms" theory was proposed, in which 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. In this theory, a TH2-cells bias against TH1-cells is important for a normal pregnancy, indicating the crucial role of the activation of maternal humoral immunity following the recognition of fetal antigens during pregnancy[4,5,6,7,8,9].

On the other hand, progress in understanding the immunologic mechanisms for the continuation of pregnancy has been made in studies of women with unexplained recurrent abortion over the past three decades. Specifically, several investigators have reported the existence of immunologically explainable recurrent spontaneous aborters, and immunotherapy for these patients using their partner's or a third party's leukocytes has been reported by several authors, including ourselves[14,15,17,18,19]. Concerning the TH1/TH2 balance in patients with recurrent abortion, Hill et al. first reported the increased TH1 cytokine production by peripheral lymphocytes exposed to JEG3 choriocarcinoma stimulation[10]. Lim et al. reported that levels of TH1 cytokines were significantly greater and higher in women with recurrent abortions compared with normal controls[11].

The results of immunotherapy for unexplained recurrent abortion indicate a correlation between the generation of immunologic humoral factors and the successful continuation of a pregnancy. The results of some case-controlled studies of immunotherapy in recurrent spontaneous abortions show that the outcome of subsequent pregnancies is significantly improved by the injection of paternal lymphocytes, as compared with that after an injection of autologous cells[25,26], although Ober et al. reported the ineffectiveness of this treatment[27]. 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[28].

Although it is still controversial as to which marker is suitable for the immunotherapy protocol in recurrent aborters, one important eligibility selection criterion for patients for immunotherapy is the presence or absence of MLR-BAbs before immunotherapy. This was emphasized by some investigators, including ourselves[15,17,18,19,20,21]. We reported that patients with negative MLR-BAbs benefit from immunotherapy with their partner's lymphocytes in both unexplained primary recurrent abortions[18] and secondary recurrent abortions[21] and also reported that signifcant MLR-BAbs were induced in almost all patients who underwent immunotherapy with their partner's lymphocytes. Thus, it is possible that the MLR-BAbs reflect the TH1/TH2 balance in patients with unexplained recurrent abortion.

In this study, we analyzed MLR-BAbs and the percentage of CD4-positive cells, TH1-cells, and TH2-cells in patients with unexplained recurrent abortions. TH1-cells were determined by the intracellular appearance of IFN-gamma, and TH2 cells were determined by the intracellular appearance of IL-4. The percentage of TH1-cells and the TH1/TH2 ratio in patients with negative MLR-BAbs was significantly higher compared with that in patients with positive MLR-BAbs. Moreover, the MLR-BE showed a significant negative correlation with the TH1/TH2 ratio.

The correlation indicates that the MLR-BAbs detected by the mixed lymphocyte culture reaction-blocking assay reflect the TH1/TH2 balance. Specifically, the positive MLR-BAbs indicate the predominance of TH2-cells, and the negative MLR-BAbs indicate the predominance of TH1-cells in patients with unexplained recurrent abortion.

To date, the relationship between the dynamic changes in MLR-BAs and TH1/TH2 balance has not been fully elucidated after immunotherapy with the partner's lymphocytes for unexplained recurrent abortion, and such analyses are considered crucial to obtain a more concrete conclusion concerning the relationship between the MLR-BAbs and TH1/TH2 balance.

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