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## Inhibitory serum factor of lymphoproliferative response to allogeneic cells in pregnancy

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**Introduction:** An inhibitory serum factor of mixed lymphocyte culture (MLC) has been associated with successful pregnancy after lymphocyte transfusion in women with unexplained recurrent spontaneous abortions (RSA). **Objective:** Investigate whether the inhibitory serum factor of MLC is essential for a successful pregnancy. **Method:** Sera from 33 healthy pregnant women and from 40 women with RSA were assessed by a one-way MLC in which the woman's lymphocytes were stimulated with her partner's lymphocytes or with third party lymphocytes. **Results:** An inhibitory serum effect (inhibition > 50% as compared to normal serum) was detected in 45% of the pregnant women who had at least 1 previous parity, in 8% of the primigravida, in 29% of those with one abortion and in 58% of those with more than one abortion. **Conclusion:** MLC inhibitory serum factor does not seem to be an essential factor for pregnancy development. Therefore, it should not be considered as a parameter for the assessment of RSA patients.

**UNITERMS:** Mixed lymphocyte culture test. Pregnancy. Habitual abortion.

### INTRODUCTION

The maternal immunologic response to the fetus has been assessed in several studies.<sup>1-4</sup> However, the essential factors that lead to the establishment and maintenance of successful pregnancy remain unclear. It has been suggested that in a normal pregnancy there is a modulation of potentially deleterious immune responses against the fetus in order to prevent rejection reactions.<sup>5,6</sup> Thus, an abnormal allogeneic response, with insufficient production of blocking factors, has been proposed as the cause of certain unexplained recurrent spontaneous

abortions (RSA).<sup>1-3,6</sup> In order to induce an adequate maternal immune response, these patients have been immunized with paternal or third party donor leukocytes.<sup>3,7-10</sup>

The mixed lymphocyte culture (MLC) is an *in vitro* model for allogeneic T cell proliferative response that has been used to detect blocking factors in maternal serum. Several investigators reported an inhibitory serum effect upon the autologous lymphoproliferative response to the partner's lymphocytes in women with successful pregnancy and its absence in RSA patients.<sup>3,5,6</sup> Therefore, it has been suggested that the absence of this factor may predispose to abortion.<sup>1,2,11</sup> Moreover, its determination has been adopted as a parameter to select<sup>3,9,11</sup> and to assess RSA patients that receive leukocyte transfusions.<sup>2,9,12-14</sup> This factor has been observed in transfused RSA patients,<sup>2,9,11,15</sup> but controversial results have been reported regarding its association with a subsequent successful pregnancy.<sup>2,10,12,13,15-17</sup>

The MLC inhibitory serum factor has been assessed mostly in non-pregnant women with a successful obstetric

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history and in RSA patients.<sup>10,11,13,14,16,18</sup> In the present study we have assessed the sera of healthy pregnant women and of non-transfused RSA patients, in order to determine whether this factor is essential to the development of a normal pregnancy and to investigate whether its absence could be considered a relevant parameter for the diagnosis and therapeutic orientation of RSA patients.

## MATERIAL AND METHODS

1. *Patients:* The study comprised 33 pregnant women with normal obstetric histories and 40 non-pregnant women with recurrent spontaneous abortions (RSA). Thirteen of the pregnant women were primigravidae and the others had 1 to 6 previous pregnancies. They were assessed in different trimesters of pregnancy. RSA was defined as three or more successive miscarriages in the first trimester of pregnancy in women without evidence of uterine abnormalities, genital-tract infections, hormonal deficiencies or autoantibodies. Chromosome abnormalities were not detected in the patients or their partners. RSA patients were classified as primary or secondary aborters on the basis of their obstetric histories. The women who had never had a viable pregnancy were considered to be primary aborters, whereas women who had already had a live birth or stillbirth with more than 20 weeks of gestation were classified as secondary aborters.

None of the women assessed in this study had a history of previous blood transfusion. Informed consent to participate in the study was obtained from all couples. This study was conducted at the Immunology Laboratory of the Allergy, Clinical Immunology and Rheumatology Division - Department of Pediatrics of UNIFESP.

2. *Mixed lymphocyte culture:* Mononuclear cells were separated from peripheral blood using a Ficoll-Hypaque density gradient. The one-way MLC test was performed with  $1 \times 10^5$  responder cells and  $1 \times 10^5$  Mitomycin C treated stimulator cells in RPMI-1640 (Sigma, St. Louis, Mo) culture medium supplemented with HEPES, L-glutamine, non-essential amino acids, sodium bicarbonate and gentamicin. The cultures were carried out in triplicates, in the presence 20% control serum (pool of male donor sera) or 20% serum being assessed for the presence of an MLC inhibitor factor. All sera were heat inactivated at 56° C for 30 minutes. The cells were incubated at 37° C in a 5% CO<sub>2</sub> atmosphere for 6 days. Eighteen hours before termination, the cultures were labeled with 2 mCi of [<sup>3</sup>H]thymidine (specific activity  $2.48 \times 10^{11}$  Bq/mmol). The cells were retrieved using an

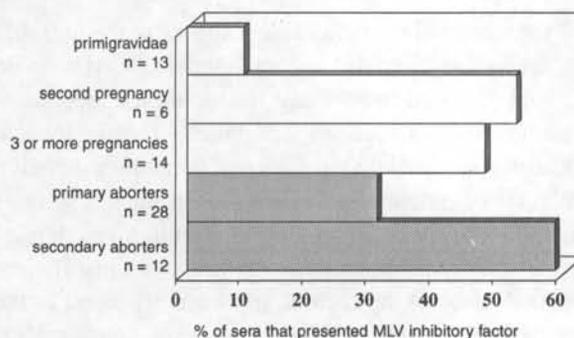
automated harvester (Titertek Cell Harvester) and processed for [<sup>3</sup>H]thymidine uptake scintillation counting. The presence of an inhibitory factor in the serum was defined as a decrease of at least 50% of the counts in cultures with the experimental serum as compared to the mean counts observed with the control serum.

3. *Phytohemagglutinin (PHA) stimulated cultures:* Peripheral blood mononuclear cells ( $1 \times 10^5$ ) were incubated in culture medium with PHA (Difco, Detroit, USA) at a concentration of 10 mg/ml, for three days. The cultures were set up in triplicates, with 20% of experimental or control sera. Otherwise, the method was the same as the one described for the MLC.

4. *Statistical analysis:* Data were analysed by the Fisher's exact test and a p value < 0,05 was taken as the level of significance.

## RESULTS

An inhibitory activity on the mixed lymphocyte culture involving the maternal lymphoproliferative response to partner's cells was observed in the sera of 45% (9/20) of the pregnant women who had at least one parity and in only 8% (1/13) of the primigravidae ( $p < 0.05$ ) (Fig. 1). The frequency of the inhibitory factor in the sera of pregnant women with one previous pregnancy (3/6) did not differ from that observed in pregnant women with more than one previous pregnancy (6/14). Fifteen patients were assessed during the first, 14 during the second and four during the third trimester of pregnancy and the serum factor was detected with the same frequency in the three trimesters of pregnancy.



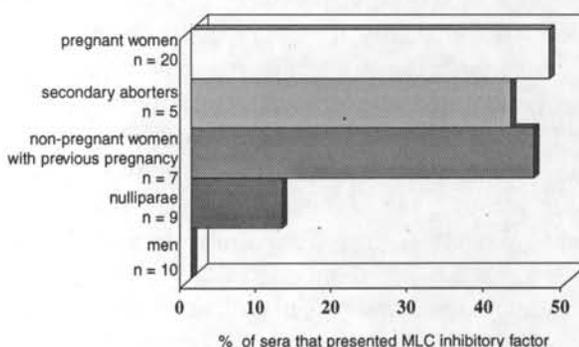
**Figure 1** - Percentage of pregnant women and patients with unexplained recurrent spontaneous abortions (RSA) that presented mixed lymphocyte culture (MLC) inhibitory serum factor. Fisher's exact test: primigravidae x 1 previous pregnancy:  $p = 0,049$ ; primigravidae x primary aborters: NS; primigravidae x secondary aborters:  $p = 0,01$ ; primary aborters x secondary aborters:  $p = 0,01$ .

Among RSA patients, 29 % of the primary and 58% of the secondary RSA patients presented the inhibitory serum factor (Fig. 1), with an overall incidence of 38%. The difference in incidence of inhibitory factor between primary and secondary aborters was not statistically significant ( $p = 0.09$ ). However, the incidence of the inhibitory factor was significantly higher among secondary ( $p = 0.01$ ), but not among primary aborters, when compared to primigravidae women. No significant differences were detected regarding the incidence of this serum factor in the primary or secondary RSA patients when they were compared to pregnant women with at least one previous pregnancy.

A suppressive effect of the serum on the maternal lymphoproliferative response to third party lymphocytes was observed in 40% of the non-primigravidae pregnant women, in 21% of the primary aborters and in 25% of the secondary aborters. With few exceptions, the sera that inhibited the response to third party cells also inhibited the response to partner's cells (Table 1).

The suppressive effect of sera from different sources was also investigated in a one-way MLC performed with cells from two HLA (human leukocyte antigens) class II incompatible donors, not including cells from the partner or from the individuals whose sera were being tested. Similar

frequencies of sera with a suppressive effect were observed among pregnant women (45%), secondary aborters (40%) and non-pregnant women with at least one previous pregnancy (43%). Only 1 out of 9 (11%) of the nulliparae sera ( $p = 0,11$  when compared to the above mentioned groups) and none of the male sera presented a suppressive effect (Fig. 2).



**Figure 2** - Percentage of sera that presented an inhibitory effect in a mixed lymphocyte culture (MLC) performed with lymphocytes from HLA class II incompatible donors.

\* with normal obstetric history.

**Table 1**  
Percentage of pregnant women with at least one previous pregnancy and women with recurrent unexplained spontaneous abortions (RSA) that presented inhibitory serum factor in mixed lymphocyte cultures (MLC) performed with different cell combinations.

Responder cells	Stimulator cells	Pregnant women n = 20	Primary aborters n = 18	Secondary aborters n = 12
F	Pm	45%	29%	58%
F	Tm	40%*	21%	25%
		40%*	14%*	25%*
P	Fm	20%	15%	22%
T	Fm	35%	8%	33%
		20%**	8%**	22%**

F - female; P - partner; T - third party  
Fm, Pm, Tm - mytomicin treated cells

\* Percentage of women who presented inhibitory serum factor both in cultures with Pm and with Tm as stimulator cells

\*\* Percentage of women who presented inhibitory serum factor both in cultures with P and with T as responder cells.

An inhibitory serum effect on the PHA-induced lymphoproliferative response was observed in only 10% (3/29) of the pregnant women, and in 5% (1/20) of RSA patients.

## DISCUSSION

The investigation of a factor in the serum of women with a successful pregnancy history and from RSA patients capable of inhibiting the *in vitro* proliferative response of maternal to paternal cells (MLC) has been the focus of many studies published over the last few years. Regarding RSA patients, the absence of this factor was considered by McIntyre et al.<sup>3</sup> as a characteristic of primary aborters for whom immunization with the partner's leukocytes would be indicated. On the other hand, according to these same authors, in secondary aborters, a group that would benefit from heparin therapy, this factor is only present in a fraction of the cases. In the present study an inhibitory activity of the serum was detected in 29% of the primary aborters and in 58% of the secondary, clearly showing that the absence of this factor cannot be used as a distinctive marker for primary aborters.

Another aspect that has drawn attention to the MLC inhibitory serum factor has been its potential to predict the outcome of a pregnancy following the immunization with parental leukocytes. Some authors have found that the development of this factor could be considered predictive of a successful pregnancy<sup>2,4,5,8,11,12</sup>, while others have not found such a correlation.<sup>10,14-16</sup> We reasoned that if this factor is biologically important for the development of a successful pregnancy, it should regularly be detected in the serum from pregnant women. The present data show that this is not the case, since the inhibitory factor was detected in only 1 out of 13 primigravidae, all of whom have already delivered a healthy baby. Furthermore, a similar incidence of the inhibitory factor was observed in pregnant women who had at least one previous parity (45%) and in secondary aborters (58%). We believe that these results, taken together, support the contention that the inhibitory factor should be regarded as an epiphenomenon of pregnancy rather than an important factor for fetal survival, as has already been suggested.<sup>5,18</sup>

The nature of the MLC inhibitory factor is not yet fully clarified. It has been suggested that the blocking activity may be related to antibodies directed against HLC antigens or against trophoblast antigens that cross-react with HLA antigens, or could represent anti-idiotypic antibodies directed to the T cell receptors (TCR) involved in allorecognition. Our study was not specifically designed to study the nature of the inhibitory factor, but we have addressed the question concerning its specificity in blocking the response of the pregnant or RSA women against their partners' cells. Results have shown that the majority of the sera that inhibited the cultures with the partner cells also showed inhibition in cultures with other cell combinations (Table 1 and Fig. 2), confirming observations of other authors.<sup>2,6</sup> It is interesting to note that no inhibitory activity was detected in any of the male

sera tested, as already reported in medical literature.<sup>6</sup> When the women's sera were assayed in PHA-stimulated cell cultures, only a few (three out of 29 pregnant and one out of 20 RSA) showed an inhibitory effect. Thus it appears that the inhibitory factor somehow interferes with allorecognition or, alternatively, that it is not powerful enough to block the response to mitogens that represent a stronger stimulus than allogeneic cells.

## CONCLUSION

Our data suggest that the MLC inhibiting factor should be regarded as a consequence of pregnancy rather than an essential factor for fetal survival. Therefore, we believe that this test, which is costly, is not informative either regarding the decision to immunize RSA women with parental leukocytes or as a predictor of a successful pregnancy in women submitted to immunization. It is therefore our opinion that the only immunological test that should be performed in RSA patients that are being considered for entering an immunization protocol is the search for lymphocytotoxic antibodies against the cells (crossmatch) that will be used for immunization. A positive crossmatch should contraindicate the immunization in order to avoid hypersensitization and also because, as noted by some authors,<sup>10</sup> a positive crossmatch is already an indication that an immune response to paternal antigens has occurred.

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## RESUMO

**Introdução:** A presença de fator sérico inibidor de cultura mista de linfócitos (CML) tem sido associada ao sucesso gestacional observado em mulheres com história de abortamento de repetição sem etiologia (AER-SED) submetidas a transfusão de linfócitos. **Objetivo:** Investigar se a presença de fator sérico inibidor de CML é essencial para o sucesso gestacional. **Método:** Soros de 33 gestantes saudáveis e 40 mulheres com AER-SED foram avaliados em CML unidirecional, em que os linfócitos das mulheres foram estimulados por linfócitos de seus parceiros ou de indivíduos não aparentados. **Resultados:** Um efeito inibidor (inibição > 50% quanto comparado com soro normal) foi detectado em 45% das gestantes a partir da 2ª gestação 8% das primeigestas, em 29% das abortadeiras primárias e, em 58% das secundárias. **Conclusão:** Este fator não parece ser elemento essencial para o sucesso gestacional. Portanto, não deve ser considerado parâmetro para avaliação de pacientes com ARE-SED.

## REFERENCES

1. Rocklin RE, Kitzmiller JL, Garovoy MR. Maternal-fetal relation: II. Further characterization of an immunologic blocking factor that develops during pregnancy. *Clin Immunol Pathol* 1982; 22: 305-315.
2. Beer AE, Semprini AE, Zhu X, Quebbeman JF. Pregnancy outcome in human couples with recurrent spontaneous abortions: HLA antigen profiles; HLA antigen sharing; female serum MLR blocking factors, and paternal leukocyte immunization. *Exp Clin Immunogenet* 1985; 2:137-153.
3. McIntyre JA, Faulk WP. Laboratory and clinical aspects of research in chronic spontaneous abortion. *Diag Immunol* 1985; 3:163-170.
4. Beer AE, Quebbeman JF, Hamazaki Y, Semprini AE. Immunotherapy of recurrent spontaneous abortion. In: Gill TJ, Wegmann TG, eds. *Immunoregulation and fetal survival*. New York : Oxford University Press. 1987:286 - 299.
5. Thaler CJ, McIntyre JA. Fetal wastage and nonrecognition in human pregnancy. *Immunol Allergy* 1990; 10:79-101.
6. Scott JR, Rote NS, Branch DW. Immunologic aspects of recurrent abortion and fetal death. *Obstet Gynecol* 1987; 70: 645-656.
7. Hill JA. Immunologic mechanisms of pregnancy maintenance and failure: a critique of theories and therapy. *Am J Reprod Immunol* 1990; 22: 33-42
8. Mowbray JF, Liddell H, Underwood JL, Gibbings C, Reginald PW, Beard RW. Controlled trial of treatment of recurrent spontaneous abortion by immunization with paternal cells. *Lancet* 1985; 15:941-943.
9. Takakuwa K, Kanazawa K, Takeuchi S. Production of blocking antibody by vaccination with husband's lymphocytes in unexplained recurrent aborters: the role in successful pregnancy. *Am J Reprod. Immunol. Microbiol* 1986;10:1.
10. Carp HJA, Toder V, Gazit E, Orgad S, Mashiach S, Nebel L, Serr DM. Immunization by paternal leukocytes for prevention of primary habitual abortion: results of a matched controlled trial. *Gynecol Obstet Invest* 1990; 29: 16-21.
11. Unander AM, Lindholm A. Transfusion of leukocyte-rich erythrocyte concentrates: A successful treatment in selected cases of habitual abortion. *Am J Obstet Gynecol* 1986; 154: 516-520.
12. Takakuwa K., Goto S., Hasegawa I. et al. Result of immunotherapy on patients with unexplained recurrent abortion: A beneficial treatment for patients with negative blocking antibodies. *Am J Reprod Immunol*1990; 23: 37- 41.
13. Sugi T, Makino T, Maruyama T, Kim K Iizuka R. A possible mechanism of immunotherapy for patients with recurrent spontaneous abortions. *Am J Reprod Immunol* 1991; 25: 185-189.
14. Illeni MT, Marelli G, Parazzini F, Acaia B, Bocciolone L, Bontempelli M et al. Immunotherapy and recurrent abortion: a randomized clinical trial. *Hum Reprod* 1994; 9:1247-1249.
15. Ho HG, Gill TJ, Hsieh HJ, Jiang JJ, Lee TY, Hsieh CY. Immunotherapy for recurrent spontaneous abortion in a chinese population. *Am J Reprod Immunol* 1991; 25:10-15.
16. Cowchock FS, Smith JB, David S, Scher J, Batzer F, Corson S. - Paternal mononuclear cell immunization therapy for repeated miscarriage: predictive variables for pregnancy success. *Am J Reprod Immunol* 1990; 22: 12 -17.
17. Clark D and Daya S. Trials and tribulation in the treatment of recurrent spontaneous abortion. *Am J Reprod Immunol* 1991; 25:18-24.
18. Coulam CB. Immunologic tests in the evaluation of reproductive disorders: a critical review. *Am J. Obstet Gynecol* 1992; 167: 1844-51.