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Interpretation of blocking activity in maternal serum depends on the equation used for calculation of mixed lymphocyte culture results

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SUMMARY

Immunosuppressive blocking factors in maternal serum are usually determined by inhibition of mixed lymphocyte cultures (MLC), but reports on the importance of these factors for successful pregnancy are conflicting. Here we measured serum blocking activity in men, non-pregnant nulliparous women, non-pregnant multiparous women, women with normal pregnancies, and in women who had had recurrent spontaneous abortions and were treated with leucocyte immunizations. Three different equations were used for calculation of blocking activity: blocking effect index (BEI); stimulation index (SI); and blocking index (BI). By all three methods of calculation, significantly lower levels of blocking activity were noted for men and women compared with pregnant women and multiparae. In the patients with a history of recurrent spontaneous abortions blocking activity as determined by BEI and BI increased into the positive range after treatment with infusions of third-party donor leucocytes in a statistically significant number of women ($P < 0{\cdot}05$). However, blocking activity as determined by BEI had a higher predictive value for successful pregnancy than did that determined by BI or SI. Our data suggest that the equation used for calculating BEI is superior to other methods for the determination of blocking activity when monitoring the response to leucocyte immunization in women with recurrent spontaneous abortion. However, these results also cast doubt on the importance of blocking antibodies in histories of recurrent abortion, since pregnancies occurred in the absence and spontaneous abortions occurred in the presence of blocking activity.

Keywords blocking activity recurrent abortion

INTRODUCTION

Circulating blocking antibodies that specifically inhibit the maternal immune response to paternal or trophoblast antigens have been proposed as one explanation for the success of normal pregnancies (Hellstrom, Hellstrom & Brawn, 1969; Rocklin, Kitzmiller & Garvoy, 1982; Scott, Rote & Branch, 1987). Unexplained recurrent spontaneous abortions have been attributed to the absence of these antibodies (Fizet & Bousquet, 1983; Rocklin *et al.*, 1976; Scott *et al.*, 1987; Stimson, Strachan & Shepherd, 1979). However, these serum factors are not well characterized, and there is no direct laboratory test to measure blocking activity *in vivo*.

Inhibition of the one-way mixed lymphocyte reaction (MLR) is the most commonly used indirect *in vitro* assay to detect blocking factors in maternal serum. However, several methods have been utilized to calculate and report the results. In this study the level of blocking activity in non-pregnant and pregnant subjects and subjects with a history of recurrent spontaneous abortion was compared using the same MLR data calculated by three different equations. The purpose was to determine which method was most useful clinically.

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SUBJECTS AND METHODS

Subjects

All subjects were evaluated in the Department of Obstetrics and Gynecology at the University of Utah Medical Center; the study was approved by the University of Utah Institutional Review Board. The subjects were divided into the following four groups: men ($n=8$); non-pregnant nulliparous women ($n=21$); non-pregnant multiparous women with previously normal pregnancies ($n=16$); and women in the first trimester with normal, ongoing pregnancies ($n=8$).

In addition, 36 women with histories of recurrent spontaneous abortion were enrolled with their partners in a prospective, randomized, double-blind protocol to evaluate the efficacy of leucocyte immunization treatment. All had at least three

consecutive first-trimester spontaneous abortions with no more than one live birth. On the basis of obstetric history, the patients were classified as primary aborters (no pregnancy progressing beyond the first trimester, 30 women) or secondary aborters (consecutive abortions by the same husband after having one live birth, six women). Anatomic, endocrine, genetic, and autoimmune abnormalities were excluded by investigation with hysterosalpingography, late luteal-phase endometrial biopsies, chromosomal analysis of both partners, and assays for lupus anti-coagulant and anti-cardiolipin antibodies (Branch *et al.*, 1989). No recurrent abortion patients had pre-immunization anti-paternal leucocytotoxic antibodies (Mittal *et al.*, 1968), and HLA-A, -B, -C, -DR, -DQ typing (Terasaki *et al.*, 1978) was performed for 30 of the 36 couples. HLA antigen sharing was as follows: none ($n=5$); ($n=3$); two ($n=9$); three ($n=6$); and four or more ($n=7$). The mean age (± 1 s.d.) for each group was as follows: men, $38{\cdot}5 \pm 7{\cdot}4$ years; nulliparous women, $31{\cdot}9 \pm 4{\cdot}6$; multiparous women, $35{\cdot}3 \pm 5{\cdot}4$ pregnant women, $31{\cdot}5 \pm 4{\cdot}3$; and recurrent aborters, $35{\cdot}8 \pm 5{\cdot}3$. The mean gravidity (± 1 s.d.) for the multiparous women was $2{\cdot}6 \pm 2{\cdot}3$ (range 1–10), for the pregnant women $2{\cdot}4 \pm 2{\cdot}3$ (range 1–8), and for recurrent abortion patients $5{\cdot}3 \pm 1{\cdot}9$ (range 3–10).

Methods

Peripheral blood was collected in heparinized tubes (Vacutainer Systems; Becton Dickinson, Rutherford, NJ) from all subjects. One-way MLRs were performed on the mononuclear cells separated by Ficoll–Hypaque density centrifugation. The cell suspensions were adjusted to 1×10^6 cells/ml and divided into stimulator and responder samples. The stimulator cells were inactivated by exposing them to 20 Gy of irradiation. The MLRs consisted of 0·10 ml of responder cells and 0·10 ml of stimulator cells in each well of a 96-well tissue culture plate (corning Laboratory Products, Corning NY). Sixteen possible combinations of responders and stimulators were set up in triplicate in a medium that consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) and contained 10% patient serum in one plate and 10% male **AB**-positive serum in the other plate. The tissue culture plates were incubated at 37°C in humidified air containing 5% CO_2 for 6 days. On the day 5, 1 μCi of ^{3}H-thymidine was added to each well. Cell cultures were harvested onto filter discs by aspiration, using a multiple sample harvester (Cambridge Technologies, Cambridge, MA). The cell-laden filter discs were then placed in polypropylene vials, and 3 ml of scintillation fluid (Packard Instrument Company, Downers Grove, IL) were added to each vial. Using a scintillation counter (LKB, Bromma, Sweden), ^{3}H-thymidine uptake by the proliferating responder cells was measured and the activity reported in ct/min.

Calculations

Serum blocking activity was calculated by each of the equations shown below. We have stated the results of all three calculations as a percentage and the stimulation index (SI) and blocking index (BI) using a one-minus equation for easy comparison with the blocking effect index (BEI). Thus, for each calculation, increasing values indicate increasing blocking activity.

In each equation P2 and C2 represent the proliferative response of the Patient's cells in response to her male partner's stimulator cells in her serum (or the female partner in the case of men) and **AB**-positive Control serum, respectively. P1 and C1 represent the background proliferation, being the Patient's cells in response to her own cells as stimulators in her serum and **AB**-positive Control serum, respectively. The indices were calculated as follows:

$$\text{BEI (\%)} = \left(1 - \frac{\text{mean ct/min of culture in patient serum (P2)} - \text{background in patient serum (P1)}}{\text{mean ct/min of culture in } \mathbf{AB}^+ \text{ serum (C2)} - \text{background in } \mathbf{AB}^+ \text{ serum (C1)}}\right) \times 100$$

This method of calculating the serum blocking activity is a modification of a method (Takakuwa, Kanazawa & Takeuchi, 1986) that we have used in our laboratory since 1986. Blocking factor activity is considered to be present if the BEI is greater than 20%, based on the mean $\pm$ s.d. of the serum blocking activity of non-pregnant nulliparous women test in our laboratory ($n=21$).

$$\text{SI (\%)} = \left(1 - \frac{\text{mean ct/min of culture in patient serum (P2)/ background in patient serum (P1)}}{\text{mean cpm of culture in } \mathbf{AB}^+ \text{ serum (P2)/ background in } \mathbf{AB}^+ \text{ serum (C1)}}\right) \times 100$$

Using this equation, blocking factor activity is considered to be present if the value is greater than 25% (Hofmeyr *et al.*, 1987).

$$\text{BI (\%)} = \left(1 - \frac{\text{mean ct/min culture in patient serum (P2)}}{\text{mean ct/min culture AB}^+ \text{ serum (C2)}}\right) \times 100$$

The presence of blocking factors is indicated by a value of greater than 20% (Smith & Cowchock, 1988).

Immunization protocol

The recurrent abortion leucocyte immunization protocol was carried out in a double-blind fashion. Depending upon the randomization, patients were infused intravenously with either their male partner's leucocytes, third-party donor leucocytes, or with a comparable volume of normal saline. For the leucocyte preparation, a one-half unit of heparinized peripheral blood was obtained from the male partner of each couple. A similar amount of blood was obtained from an unrelated, third-party donor in cases randomized to receive donor leucocyte infusions. All blood samples used for leucocyte infusions were negative for erythrocyte antibodies, hepatitis B surface antigen and core antibody, elevation of alanine aminotransferase, antibody to HIV-I and III, and syphilis serology. **ABO** incompatibility did not exclude the couples from leucocyte immunizations; when indicated, Rh-negative women were treated with Rh-immune globulin immediately prior to the leucocyte infusion. The leucocytes were separated under aseptic conditions by differential centrifugation (COBE 2991 Blood Cell Processor, Coulter Electronics, Hialeah, FL) in the University of Utah Hospital Blood Bank.

Patients were draped so they could not observe the peripheral vein infusion by the nurse clinician. The same treatment was repeated after 1 month, and all patients were encouraged to attempt pregnancy after the second infusion. MLRs were tested before and 1 month after completion of treatment. Of the immunized women who became pregnant, five were treated with paternal leucocytes (four primary and one secondary aborter), and 11 had received third-party leucocytes (10 primary and one

secondary aborter). Only the 16 women who actually received leucocytes are included in this analysis.

Statistical analysis

Neither the actual indices or the logs of the indices were normally distributed. Hence, the Kruskal–Wallis non-parametric analysis of variance was used to compare the blocking effects in the six groups of subjects (Feïnstein, 1977). Pairs of groups were compared by multiple comparison methods, based on the Kuskal–Wallis rank sums (Feinstein, 1977). The paired Student's *t*-test and the sign test were used for the paired numerical data. For the categorical data, the two-tailed Fisher's exact test was performed after construction of two-by-two contingency tables (Sargent, Wilkins & Redman, 1988).

RESULTS

Table 1 shows the mean (±s.e.m.) and positivity (presence) of blocking factors according to each equation. Significantly lower mean values were found using the sera of the men and non-pregnant nulliparae compared with multiparous women by the BEI and BI methods of calculation. The SI detected a significant difference only between the non-pregnant nulliparae and the non-pregnant multiparae. No equation showed a significant difference in mean values or positivity for serum blocking factors between non-pregnant nulliparous women and recurrent spontaneous abortion patients prior to the leucocyte infusions. Using the SI equation, three (37·8%) men and four (19%) non-pregnant nulliparous women allegedly had blocking activity in their serum, while all men were negative for blocking activity and only one (4·8%) nullipara was positive with the BEI and BI equations.

Figure 1 shows the serum blocking activity before and after paternal or third-party leucocyte immunization according to success (live birth) of the subsequent pregnancy. Using the BEI equation, an upward trend of values was noted in all cases: seven

Table 1. Serum blocking activity according to three different methods of calculation. The comparisons of number positive for each equation have not been corrected for effects resulting from multiple testing

		Blocking effect index*			Stimulation index†			Blocking index‡		
Group	Total subjects	Mean+s.e.m. (%)	Positive *N*	Positive %	Mean+s.e.m. (%)	Positive *N*	Positive %	Mean+s.e.m. (%)	Positive *N*	Positive %
(A) Men	8	−554·8+380·9	0	0	193·7+68·7	3	37·5	300·7+81·0	0	0
(B) Non-pregnant nulliparae	21	−71·2+19·6	1	4·8	155·5+20·8	4	19·0	164·5+14·8	1	4·8
(C) Non-pregnant multiparae	16	53·7+14·9	13	81·3	47·2+12·9	14	87·5	70·0+11·3	12	75·0
(D) Pregnant women	8	17·4+9·3	3	37·5	67·9+14·1	5	62·5	87·4+9·1	3	37·5
(E) Primary recurrent abortion	30	−148·3+60·6	5	16·7	175·0+56·2	11	36·7	199·5+36·1	3	10·0
(F) Secondary recurrent abortion	6	−63·3+52·4	1	16·7	168·7+61·2	2	33·3	153·0+42·4	1	16·7

* $P<0·05$ (A–C, B–C, and C–E using mean value; A–C, B–C, B–D, C–D, C–E and C–F using positivity).
† $P<0·05$ (B–C and C–E using mean value; A–C, B–C, B–D, C–E, and C–F using positivity).
‡ $P<0·05$ (A–C, B–C and C–E using mean value; A–C, B–C, B–D, C–E and C–F using positivity).

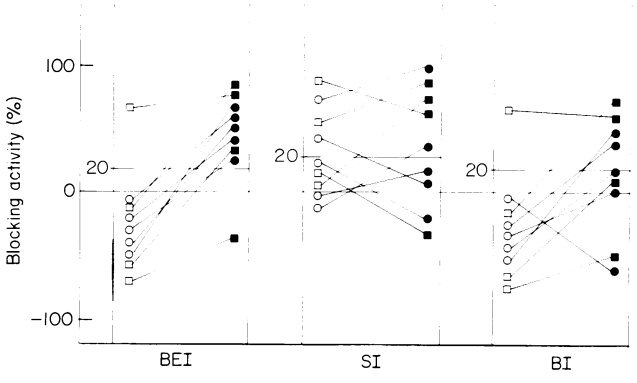

Fig. 1. Serum blocking factor activity before and after leucocyte infusions in patients with successful pregnancy outcomes. For each graph, the cutoff between negative and positive blocking activity is indicated by the solid line. (*P* value by two-tailed Fisher's exact test). BEI, blocking effect index; SI, stimulation index; BI, blocking index. ○, donor cell pre-immunization; ●, donor cell post-immunization; □, paternal cell pre-immunization; ■, paternal cell post-immunization.

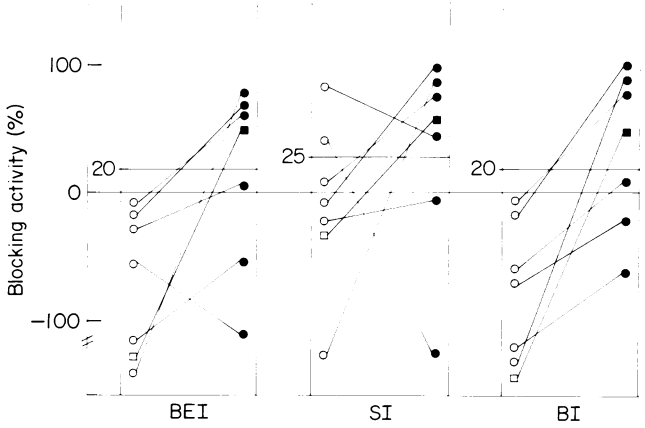

Fig. 2. Serum blocking factor activity before and after leucocyte infusions in patients with unsuccessful pregnancy and outcomes. For each graph, the cut-off between negative and positive blocking activity is indicated by the solid line. (*P* value by two-tailed Fisher's exact test). BEI, blocking effect index; SI, stimulation index; BI, blocking index. ○, donor cell pre-immunization; ●, donor cell post-immunization; □, paternal cell pre-immunization; ■, paternal cell post-immunization.

out of the eight subjects in the negative became positive for blocking activity ($P = 0{\cdot}07$ by the sign test). The actual change in blocking activity was significantly increased using the BEI equation ($P = 0{\cdot}003$, Student's *t*-test), but the increase was not significant for either the BI or SI equation. Post-immunization upward trends were noted in the majority of patients using the BI method of calculation, but the values were still below those accepted for blocking activity in five out of the nine patients. The SI values demonstrated no consistent trend. The results were also the same when the data were calculated for the primary aborters alone.

Figure 2 displays the same information for the patients who had another spontaneous abortion in their next pregnancy. It is apparent from these data that the post-immunization trends and levels of blocking activity were less consistent and correlated poorly with pregnancy outcome. Indeed, blocking activity was present in many patients according to all methods of calculation even though the pregnancies ended with spontaneous abortion.

Table 2 compares the predictability for pregnancy outcome according to each equation using post-treatment levels of serum blocking activity. Using live births as the predicted outcome, the presence of blocking activity as calculated by the BEI method demonstrated the highest sensitivity (89% *versus* 56% for the SI and 44% for the BI). The specificity of the BEI was as good as for either of the other two methods of calculations (43% for BEI *versus* 33% for SI and 43% for BI). In addition, the BEI equation provided the lowest value in the false-negative rates. Consequently, blocking activity as calculated by the BEI method had the highest positive predictive value for live births, at 67%, while the SI and BI methods had positive predictive values of 56% and 50%, respectively.

Table 2. Predictability of successful pregnancy outcome (live birth) after leucocyte immunization according to three different methods of calculating maternal serum blocking activity

	Blocking effect index	Stimulation index	Blocking index
Sensitivity	0·89	0·56	0·44
Specificity	0·43	0·33	0·43
False-negative	0·25	0·67	0·63
False-positive	0·33	0·44	0·50
Positive predictive value (%)	0·67	0·56	0·50
Negative predictive value (%)	0·75	0·33	0·38

DISCUSSION

Although the mechanisms that prevent rejection of the conceptus are incompletely understood, it has been suggested that maternal immune aberrations may be related to repeated abortions. This has become a controversial area, and attempts to treat idiopathic recurrent spontaneous abortions with various immunotherapeutic regimens have generated a great deal of publicity (Slapsys & Clark, 1980; Mowbray *et al.*, 1985; Adinolfi, 1986; Takakuwa *et al.*, 1986; Hofmeyr *et al.*, 1987; Smith & Cowchock, 1988).

Cells with suppressive properties are reportedly present in the endometrium and decidua during normal pregnancies and absent in early pregnancy failure, but currently there is no convenient or practical clinical test for measuring these cells (Caudle *et al.*, 1983; Clark, 1985; Daya *et al.*, 1985). We (Caudal *et al.*, 1983) and others have found no correlation between the success of pregnancy and HLA histoincompatibility between couples (Gill, 1983; Kilpatrick, 1984; Oksenberg *et al.*, 1984; Beer *et al.*, 1985; Unander & Lindholm, 1986; Regan & Braude, 1987; Smith & Cowchock, 1988) or between the presence or absence of anti-paternal leucocytotoxic antibodies (Zak & Good, 1959; Gill, 1983; Mowbray *et al.*, 1985; Smith & Cowchock, 1988). Consequently, no further analysis of these data was carried out in the present study.

Table 3. Summary of equations used for calculating blocking activity

	*Term used and structure of equations for calculating the blocking factors	Criteria for presence of blocking factors (positivity)
Beer *et al.* (1981)	SI = (C2 or various combination)/CL	
Mowbray *et al.* (1985)	SI for all conditions (P2/P1) (C2/C1)	
Oksenberg *et al.* (1983)	SI = C2/C1, RR = (C2 − C1)/(C3 − C1) × 100	SI > 3, RR/20%
Thomas *et al.* (1985)	SI = C2/C1, RR = (C2/C1)/(C3 − C1)	SI > 3 or RR > 30%
Labarrere *et al* (1986)	SI = P2/P1, C2/C1	
Nicholas & Panayi (1986)	Expressed as means of P2 and C2	Mean of P2 < (Mean if C2–1 s.d.) in each MLC
Takakuwa *et al.* (1986)	BI = (1 − P2/C2) × 100	More than 22%
Unander *et al.* (1986)	BI = (P2/C2) × 100	Mean of P2 < mean of C2 in each MLC by Mann–Whitney *U*-test
Hofmeyr *et al.* (1987)	SI = (P2/P1)/(C2/C1) × 100	Less than 75%
Sargent *et al.* (1988)	BI = P2/C2	
Smith & Cowchock (1988)	BI = P2/C2	Less than 80%
Gatenby *et al.* (1989)	RR = (C2 − C1)/(C3 − C1) × 100	
Park *et al.* (current study)	BEI = 1 − (P2 − P1)/(C2 − C1) × 100	More than 20%

SI, stimulation index; C, control serum; P, patient serum; RR, relative response; BI, blocking index; BEI, blocking effect index.

1, Mean ct/min of MLC of patient's cell as responder and as stimulator (autologous background); 2, Mean ct/min of MLC of patient's cell as responder and husband's cell as stimulator; 3, Mean ct/min of MLC of patient's cell as responder, and pooled cell or unrelated (or third-party) cell as stimulator.

* Reclassified by authors.

Serum components that can inhibit cell-mediated immune function *in vitro* include receptor blocking antibodies; blocking alloantibodies; and non-specific blocking factors (Slapsys & Clark, 1980). Receptor blocking antibodies include anti-T cell idiotype antibodies and antibodies to Fc receptor- associated antigen. Blocking alloantibodies are non-cytotoxic alloantibodies that block T cell recognition of graft/donor antigens. Non-specific blocking factors include immunoregulatory alphaglobulins, interleukin-2 inhibitors, prostaglandins, and very-low-density lipoproteins. It is possible that individual components of the MLR are important in pregnancy success, and these and the kinetics of the MLR deserve further investigation. Nevertheless, studies of the relationship of circulating maternal serum inhibitory factors as measured by MLR to successful pregnancy and to recurrent spontaneous abortion are conflicting. Some investigators report that leucocyte immunization treatment of recurrent aborters is successful only if the patient initially has no serum blocking activity but develops it after treatment (Holland & Holland, 1966; Mowbray *et al.*, 1985; Takakuwa *et al.*, 1986). Others have found no correlation between pregnancy outcome and the presence of serum blocking activity, either before or after immunization (Gill, 1983; Smith & Cowchock, 1988). Some have cast doubt on the entire concept of circulating blocking factors, because blocking activity often does not appear until late in the first or second trimester of the first pregnancy (Scott *et al.*, 1987); agammaglobulinaemic women have normal pregnancies (Faulk *et al.*, 1974; Kobayashi, Hyman & Stiehm, 1980; Roger, 1985); and because animals rendered incapable of producing immunoglobulins or mounting a humoral immune reponse also have successful pregnancies (Roger, 1985). It has also been shown that the blocking activity present during a normal pregnancy is not always specific for the male partner's stimulator cells (Holland & Holland, 1966) and may be directed at trophoblast antigens (Beer *et al.*, 1981; McIntyre & Faulk, 1986).

Adding to this confusion, the published techniques for measuring serum blocking activity by means of MLR have not been standardized and, as illustrated in Table 3, there is no universally accepted method for calculating or reporting the results. Therefore, there is no absolute cut-off value for determining a positive result; rather, the levels are empirically defined by each investigator. In addition, there is a substantial variability in the MLR assay itself, and the reproducibility and sensitivity of MLR inhibition differs between laboratories (Hollander & Wolfe, 1973; Oksenberg *et al.*, 1983; Gill, 1983; Mowbray *et al.*, 1983, 1985; Thomas *et al.*, 1985; Nicholad & Panayl, 1986; Labarrere *et al.*, 1986; Hofmeyr *et al.*, 1987; Burlingham, 1988; Smith & Cowchock, 1988; Gatengy *et al.*, 1989).

The lack of uniformity in the laboratory techniques used (Oksenberg *et al.*, 1983), in nomenclature, in the equations utilized, and interpreting the results of MLR assays has undoubtedly contributed to the conflicting views on their clinical usefulness and correlation with successful pregnancy. It is for these reasons that we compared the results of the same MLR data from our own patients using three different equations to determine the presence or absence of blocking effects. Our results indicate that post-immunization blocking activity in maternal serum, as measured by any of the methods, is not an absolute requirement for a successful pregnancy; conversely, blocking activity was present according to all methods in some patients who had subsequent abortions. However, the values obtained by the BEI equation were the closest to those that would be expected for each of the groups studied. The BEI method was also the most reliable predictor of success in subsequent pregnancies following leucocyte immunization spontaneous abortion patients.

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