Characterization of Mixed lymphocyte reaction blocking antibodies (MLR-Bf) in human pregnancy

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Abstract

Background: It is known that during normal pregnancy and after immunotherapy blocking antibodies are developed, these antibodies inhibit mixed lymphocyte reaction (MLR) and are also anti-mitogenic in nature. Mixed lymphocyte reaction blocking antibodies (MLR-Bf) are specific to the husband's lymphocytes. In the present study an attempt has been made to characterize the MLR-Bf in normal pregnancy and in recurrent spontaneous abortion (RSA) women after immunotherapy.

Methods: Serum was obtained from women of different gestational windows of pregnancy (Ist, IInd, IIIrd trimesters and post delivery period of normal pregnancy), RSA women from pre and post immunization and healthy (male and females) controls were screened for the presence of MLR-Bf. The standard mixed lymphocyte reaction (MLR) technique was used to evaluate the inhibitory effect of serum in the mixed lymphocyte reaction. Each serum was tested for cytotoxic antibodies. IgG antibodies were isolated according to the standard protocol.

Results: In the present study we have observed that there was significant inhibition of proliferation response when IgG from different trimesters of pregnancy were added into one way MLR or to phytohemagglutinin (PHA) activated lymphocyte proliferation assay (LTT). When IgG isolated from adequately immunized RSA women similar pattern was seen. It was further confirmed that amongst all the isotypes of Immunoglobulin G, only IgG3 was found to be positive for the inhibitory effect.

Conclusions: Present study indicates that MLR-Bf is IgG 3 in nature and is developed during pregnancy and also after immunotherapy in RSA women who subsequently have the successful pregnancy.

Background

Introduction

Recurrent spontaneous abortion (RSA) is defined as the loss of three or more consecutive pregnancies prior to 20 weeks of gestation. In large number of patients the underlying cause of pregnancy loss often remains unclear (**1, 2**). This may be due to anatomical uterine defects, chromosomal defects, parental chromosomal rearrangements, gene mutations, endocrine factors, sub clinical infections, environmental toxins, collagen vascular diseases, auto immune factors, and psychological trauma or stress. However, in most of the women (1% - 2%) who experience recurrent miscarriage, no cause can be identified. Alloimmune mechanisms that prevent mothers from developing immunological responses essential for the survival of the semiallogeneic pregnancy have been proposed as the cause of 50% of all such losses. The maternal recognition of paternally derived foetal antigens has been well documented (**3**), and the presence of circulating antipaternal antibodies provides unequivocal evidence of a maternal immune response to the allogenic pregnancy. In contrast to allograft transplantation, paternal histocompatibility antigens expressed on the placenta elicit only limited T cell immuno-reactivity (**4**). The immunoglobulins generated during pregnancy have been characterized as asymmetrically glycosylated antibodies (**5,6,7**). We have reported in our earlier study that significant MLR-Bf production by paternal lymphocytes in RSA women, leads to successful pregnancy (**8**). In the present study an attempt has been made to characterize the MLR- Bf in the total IgG fraction from different gestational windows of pregnancy and also in recurrent spontaneous abortion patients before and after immunization against their husband's lymphocytes.

Material and Methods

Serum samples were obtained from the individuals of different groups (**Table-1**). All gave their consent to participate in the study, and the protocol followed was after the ethical clearance by the institute. These groups included 40 women of different stages of pregnancy (Ten each in Ist, IInd, IIIrd trimesters and post delivery period), 20 RSA women (10 each of pre and post immunization), 10 healthy males and 10 unmarried females. All these were screened for the presence of MLR blocking antibodies. Serum samples were separated from non heprinized peripheral blood under aseptic conditions further these samples were heat inactivated. After heat inactivation each serum sample was aliquoted. One aliquot was added to a panel of three peripheral blood lymphocytes (PBL) activated by PHA

(phytohemagglutinin). Second aliquot was added to one way mixed lymphocyte reaction (MLR). The dilution factor used was 50% volume by volume.

Immunological parameters

Peripheral blood lymphocytes (PBL) were prepared by density gradient centrifugation on ficoll-hypaque. PBL were incubated in plastic dishes at 37°C, 5% CO_2 for 12 hrs then passed through nylon wool columns, and mainly T cells were obtained. Responders and stimulators from unrelated individuals were chosen so that there was at least one HLA class I and one HLA DR antigen mismatch was found between them. Irradiated stimulator cells (2800 rads) were cultured in round bottomed 96 well plates with the responder cell in a ratio of 1:1 and a concentration of 10^6 cells/ml. Plates were kept at 37°C in a 5% CO_2 atmosphere. Proliferation was measured at day 5 with (H^3) thymidine incorporation in the last 18 hrs before harvesting. The percentage (%) of inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{Cpm in test MLR}}{\text{Cpm in control MLR}} \times 100$$

Cpm = count per minute

MLR = mixed lymphocyte reaction

Cytotoxic antibody determination:

The percentage of Panel reactive antibodies (PRA) was evaluated against a panel of 50 individuals at room temperature using the standard NIH lymphocytotoxicity test. Sera were also tested on autologous cells to determine auto reactivity, and pretreated with dithiothreitol to differentiate IgG from IgM mediated cytotoxicity. A standard microcytotoxicity test was used to determine cytotoxicity against specific target cells. These cells were same which were used as stimulators in the MLR or in the PHA stimulation test. The same method was used to determine presence of cytotoxic antibody in pooled IgG and subclasses of IgG. 10mg/ml up to the dilution of 1: 128 was used in each test. If the sera contained lymphocytotoxic antibodies these antibodies were absorbed using platelets.

Preparation of IgG and its subclass from patient's sera:

Ten individuals each from Ist, IInd and IIIrd trimester, post delivery and post immunotherapy were taken from those subjects who were positive for MLR-Bf activity. Each serum was centrifuged for 20 min at 10,000xg. An ammonium sulphate saturated solution

was added to the serum (60%w/v) and incubated for 2 hrs so that IgG was completely precipitated. After centrifugation for 20 min at 10,000xg the pellet was resuspended in ammonium bicarbonate buffer and dialyzed against the same buffer. The serum was separated and purification of IgG was carried out. IgG was also isolated from 10 males, 10 RSA women and 10 non-pregnant unmarried females (who had no MLR-Bf activity). IgG fraction was prepared independently from each individual. Dialyzed product was fractionated on protein A columns.

Purity of the preparations was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with standard molecular weight marker and purified human IgG as well as enzyme linked immunosorbant assay (ELISA) by using monoclonal antibodies directed against IgG. Further all the isotypes of IgG (IgG1, IgG2, IgG3 and IgG4) were separated on a protein A Sephrose CL-4B column. ELISA was used to detect the IgG isotypes in each peak. Monoclonal antibodies directed against IgG1, IgG2, IgG3 and IgG4 were used in each ELISA. Each IgG fraction was lyophilized and the protein content was measured.

Statistical tests:

Results were expressed as median and interquartile range (IQR). To compare proliferation indices between different groups, the Mann-Whitney test was employed as the sample size was small in each group. However data from individual experiments were analysed by ANOVA and Bonferroni's multiple comparison test. A value of $p<0.05$ was considered statistically significant.

Results:

Effect of neat serum on Lymphocytes

To evaluate the inhibitory effect of serum we carried out some independent experiments with each serum from different groups as mentioned in the material methods section. Serum was added at 0 hrs of culture. All the serum samples which inhibited the peripheral blood lymphocytes (PBL) proliferation in primary MLR were labeled as sera containing MLR -Bf. The significance of inhibition of the mixed lymphocyte reaction were compared against normal pooled AB (non cytotoxic) serum. The differences were statistically significant ($P<0.001$).

On the basis of this inhibitory mechanism, we further investigated the effect of all the sera on PHA stimulated cells of the person's own PBL and unrelated PBL separated from three normal male controls. These were also used as stimulators in the MLR assay. All the sera having inhibitory activity inhibited unrelated PBL responses (2456 ±250 cpm) but failed to inhibit their own cellular response to PHA (30, 462 ± 1562 cpm). This indicated that inhibitory effect was not against autologous cells.

Further, to examine the inhibitory effect of purified IgG we treated half of the stimulator cells with the inhibitory IgG for 4 hrs at 4°C and washed the cells for three times, mixed with untreated homologous stimulator PBL cells before using them in MLR. Pre incubated stimulator cells with the patient's sera were added to fresh stimulator PBL cells in the ratio of 1: 1 in MLR cultures. Under these conditions, values of (H^3) thymidine uptake after 50/50 mix of pre-incubated and freshly isolated stimulator cells (32506±3217 cpm) were not different from normal controls (33617±2527cpm, $p > 0.05$). In another set of experiments, after the addition of cells, harvesting was carried out at 2nd and 4th day from the primary MLR cultures to allogenic T cell lines, the allogenic cell line proliferations were 21973±1819 cpm and 20521±2017cpm.These results indicated that the inhibition of proliferation in MLR by women's sera is not due to the generation of suppressor cells.

Determination of the inhibitory fraction in pregnant women and post immunotherapy sera

We further examined the blocking activity present in IgM and IgG fractions.The results of percent inhibition by women's sera (WS) IgG and IgM on primed and unprimed cell proliferation to alloantigens and PHA was analyzed. WS IgG inhibited autologous cell proliferation in the MLR system by 68.3±3.19%. Inhibition was found when we used WS to check the third party proliferation (59±3.08%) but to a lesser extent and failed to inhibit autologous cell proliferation (3.6±4.16%). Fresh PBL proliferation to PHA was inhibited by WS IgG to 61.3±7.4%. WS IgM did not possess any inhibitory effect on both MLR and PHA stimulations. These results revealed that the antimitogenic effect was present in the IgG and not in the IgM fraction. To further determine whether cytotoxic antibodies were responsible for the potent antimitogenic effect present in the sera, we compared the inhibitory effect before and after removal of lymphocytotoxic antibodies against HLA class I antigens by platelet absorption. These were removed in 5 WS, 3 from different trimesters of pregnancy

and 2 from RSA women after post immunotherapy. The platelet-adsorbed sera (AS) were tested independently on autologous proliferation to alloantigens from control (husband's AS) stimulator cells. They were also tested on the same donor (AS) cellular proliferation to PHA.

Inhibitory action of IgG and its isotypes on cell function

IgG and its each isotype purified from pooled sera from 5 individuals were subjected to lymphocytotoxicity and blocking activity. Pooled IgG1, IgG2 and IgG3 fractions contained significant lymphocytotoxic activity at a serial dilution of 1:64 (neat concentration 10mg/ml) against a panel of 50 T cells and 50 B cells. It was observed that IgG1 and IgG2 showed 50-80% cytotoxicity respectively. However, in IgG3 fraction it was only 3%. After determination of these lymphocytotoxicity, IgG1, IgG2, IgG3 and IgG4 were further individually tested for blocking activity.

When fractionated IgG and its isotypes (IgG1, IgG2, IgG3 and IgG4) from different stages of pregnancy (Ist, IInd, IIIrd trimester and post delivery period) and RSA women (pre and post immunization) were added (10-50 μg/μl) at 0 hr of culture and measured its inhibitory activity against the base line data (results not shown). Our results (**Table 2 and 3)** revealed that PHA and MLR proliferation was significantly inhibited ($F=56.2$, $P<0.001$), in the presence of whole IgG and IgG3 separated from all the stages of pregnancy (Ist, IInd and IIIrd trimester) and post immunized RSA women. No inhibition was found with the addition of IgG and its each isotypes fractionated from pre immunized RSA women, normal male and normal unmarried non pregnant females.

Determination of antibody 2 and antibody 3 activity in the inhibitory IgG3 fraction

Since IgG3 fraction did not contain significant cytotoxic reactivity but had a significant antimitogenic activity it was tested for its capacity either to block or to enhance the antibody I from IgG1, IgG2 and IgG4 fractions that contained antibody I reactivity. The addition of three serial dilutions of IgG3 did not change the lymphocytotoxic titers present in either IgG1, IgG2 or IgG4 fractions against a known panel of T and B cell targets (data not shown) which demonstrates absence of anti- idiotypic activity in the pooled IgG3 fraction.

Discussion

The present study was carried out to characterize the MLR-Bf present in normal pregnancy and also developed after immunotherapy. To test this we isolated IgG fractions

from women having MLR-Bf in their sera (normal pregnancy) and from post immunized RSA women having MLR-Bf in their sera and added to the cells stimulated by PHA. The same sera were also added to one way mixed lymphocyte reaction (MLR). Our initial results demonstrated that the proliferation of cells was significantly less in the presence of this fraction. To find out the specific isotype of IgG which may be inhibitory in nature we isolated and purified various subclasses of IgG. These fractions were again added to the PHA activated LTT's and also to one way MLR. Significant inhibition in the proliferation was observed ($F=56.2$, $P<0.001$) in the presence of IgG3 in comparison to IgG1 and IgG2. Our results show that blocking activity is targeted to the unprimed T cell response which is due to alloantigens. The antimitogenic effect is most likely due to the binding with foreign HLA class II antigens present on cells.

However, it is difficult to ascertain the exact site of action of the inhibitory IgG fraction. These antibodies are very likely synthesized systemically and act at the local level (placenta), blocking placental antigens and preventing immunological attack by maternal natural killer cells and cytotoxic lymphocytes. It has been reported that women of recurrent spontaneous abortion had significantly lower levels of asymmetric IgG as compared to controls (**9,10**). These antibodies show suppressive mechanism hence may be of the same nature as the blocking antibodies. It has been shown by a number of investigators that MLR-Bf is present during normal pregnancy while it is absent or very low in RSA women (**11,12,13**). It is argued that these factors may play a protective role perhaps by blocking the damaging effect of maternal lymphocytes

Some investigators have questioned the role of blocking antibodies in the maintenance of normal pregnancy (**14,15**). Their studies have suggested that alterations in the ratio of Th1 cells to Th2 cells play an important role in immune modulation. Other studies have demonstrated the indication of asymmetric IgG synthesis by a progesterone induced blocking factor (PIBF) (**16**). PIBF has been shown to influence the balance of T helper 1 cells to T helper 2 cells which subsequently alters cytokine ratios and decrease the cell mediated immune response during pregnancy (**17**). Thus the production of PIBF early in pregnancy may play a role in activation of cellular and humoral immune system to prevent the immunologic attack. However, the exact role is still controversial.

Tamura et al. (11) have reported that the presence of MLR-Bf during the first pregnancy makes a significant contribution in the success of 2nd pregnancy. **Ramhorst et al.**

(**12**) have shown that the blocking factor plays an important role in RSA women undergoing immunotherapy. We have recently demonstrated that MLR-Bf developed with the husband's lymphocytes, during pregnancy and post immunotherapy in RSA women, is important for the success of human pregnancy (**8**). **Gatenby (18)** have reported that development of MLR-Bf might be because of prolonged maintenance of CD8+ suppressor T cells, which suppress the immune mechanisms that are required for the maintenance and the outcome of successful pregnancy. Our results definitely show that the presence of MLR-Bf during pregnancy and after immunotherapy is a good prognostic marker for the success of pregnancy. The success of pregnancy in the face of potential maternal immune reactions has been largely attributed to the placenta, which appears to serve as an immunological barrier. The ability of the trophoblast tissue to survive in the conditions of allograft rejection was initially attributed to the non-antigenic nature but it was soon shown that the trophoblast expresses MHC antigens on the surface (**19**). This can lead to alloantibody formation during normal pregnancy. Alloimmunity has been indicated in several studies by the association of habitual abortion with an increased sharing of human leukocyte antigens that may prohibit the mother from making blocking antibodies (**20,21**).

Our results indicate that a significant amount of blocking antibodies developed during pregnancy and also after paternal lymphocyte immunotherapy in RSA women. The blocking factor is of IgG 3 nature, which may play an important role in the success of pregnancy in these women. **Zenclussen et al.** (**10**) have demonstrated that women undergoing immunotherapy expressed higher percentage of asymmetric IgG antibodies, which play a protective role during pregnancy.

It has been documented that inhibition of T cell activation is by the antibodies against the α-3 domain of the human MHC class I molecule. **Stach and Rowley** (**22**) have demonstrated that the inhibitory fraction is associated with TGF-β. They have suggested that immunoglobulin TGF-β conjugate is taken up by the antigen – presenting cells by FC-dependent mechanisms. It is conceivable that the inhibitory IgG preparations employed in our study contain antibody – cytokine conjugates that target and are internalized by antigen presenting cells following binding to MHC encoded public epitopes of class I or II molecules expressed on the allogenic cells. It may be postulated that absence of these inhibitory antibodies in RSA women may prevent the generation of suppressor systems, which are required for the survival of the foetus as an allograft.

Conclusion

Our results clearly demonstrate that IgG3 (approximately 25 kDa) fraction is generated during pregnancy but is absent in RSA women, unmarried non-pregnant females and normal healthy males. Upon immunotherapy IgG3 fraction is developed in RSA women who play an important role in the maintenance of pregnancy.

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Competing interests

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Author's Contributions

1. Drafted and Planned the study, Carried out all the experiments and Statistical analysis
2. Sequence alignment
3. Conceived the study and participated in its design and coordination

Table 1: Demographic profile

Different group of subjects	Number	Age (median; IQR)	Week of current pregnancy (median; IQR)	Week of abortion (median; IQR)
Ist trimester	10	26; 17-31	10; 6-11	Nil
IInd trimester	10	29; 20-30	19;12-24	Nil
IIIrd trimester	10	28; 27-32	29; 23-29	Nil
Post delivery period	10	27; 21-27	Nil	Nil
Pre immunized RSA Women	10	23; 21-30	Nil	8; 5-11
Post immunized RSA Women	10	24; 21-31	Nil	12; 8-16
Healthy males	10	25; 19-28	Nil	Nil
Unmarried females	10	24; 20-28	Nil	Nil

* Values are expressed in median and interquartile range (IQR)

Table 2: Stimulation index (SI) of MLR-Bf significant IgG and isotypes

Different group of subjects	Proportionate change in the SI of IgG and its isotypes in terms of interquartile range (IQR)				
	IgG	**IgG1**	**IgG2**	**IgG3**	**IgG4**
Ist trimester	25(14-27)	76(24-80)	68(42-82)	9(7-22)*	47(28-69)
IInd trimester	29(11-31)	82(44-84)	51(39-68)	7(6-34)*	79(65-92)
IIIrd trimester	35(17-38)	68(37-79)	69(57-82)	11(5-33)*	71(38-102)
Post delivery period	42(11-45)	57(39-61)	47(37-61)	41(23-57)	54(46-81)
Pre immunized RSA Women	38(28-56)	43(26-63)	64(29-71)	37(26-69)	66(33-87)
Post immunized RSA Women	21(19-28)	62(49-83)	72(39- 77)	8(5-24)*	52(37-69)
Healthy male sera	52(46-71)	57(39- 69)	48(34-56)	61(46-71)	41(39-58)
Unmarried females	83(39-86)	54(49-67)	65(43-73)	49(40-78)	61(43-99)

Values given in the table are calculated as stimulation index (SI) against the base line measurement and shown as median and interquartile range (IQR), n= 10 . * $P<0.001$.

Table 3: Percentage inhibition (%) of MLR-Bf significant IgG and its subclasses

Different group of subjects	Proportionate change in percent inhibition of MLR-Bf significant IgG and its isotypes in terms of interquartile range (IQR)									
	IgG		IgG1		IgG2		IgG3		IgG4	
	W x H (1:2)	H x W (2:1)	W x H (1:2)	H xW (2:1)	WxH (1:2)	HxW (2:1)	WxH (1:2)	HxW (2:1)	WxH (1:2)	HxW (2:1)
Ist trimester	39 (35-67)	26 (21-63)	22 (18-28)	24 (15-26)	17 (-11- 37)	21 (7-42)	68 (61-68)*	64 (60-63)*	19 (17-45)	17 (11-21)
IInd trimester	42 (28-71)	39 (35-64)	18 (15-24)	17 (14-19)	12 (3-33)	11 (9-15)	61 (63-70)*	62(61-67)*	29 (-14 - 33)	4 (-17 - 8)
IIIrd trimester	27 (21-62)	35 (27-67)	-14 (-27-9)	13 (-8 - 27)	15 (9- 23)	17 (14-21)	67 (61-82)*	71(62-76)*	15 (15-28)	13 (9-16)
Post delivery period	44 (20-68)	25 (23-68)	17 (-3- 20)	15 (10-30)	5 (-9 - 19)	11 (7-31)	47 (14-47)	41(23-52)	8 (4-17)	11 (6-22)
Pre immunized RSA Women	34 (28-47)	28 (23-61)	6 (-12 - 9)	-6 (-11 - 2)	2 (24-11)	5 (-22 - 7)	43 (17-37)	38(21-36)	6 (-12 - 6)	8 (5-19)
Post immunized RSA Women	51 (44-69)	60 (57-73)	-7 (-16- 7)	4 (-12 - 6)	12 (6-31)	21 (11-29)	67 (64-82)*	67(62-71)*	21 (19-26)	15 (10-30)
Healthy male sera	-9 (-21 - 2)	-17 (-8 - 4)	-17 (-19 - 2)	-8 (-11 - 3)	-15 (-27 - 5)	4 (-17 - 2)	-11 (-19 - 9)	-12(-18-7)	-6 (-29 - 4)	-11 (-18 - 19)
Unmarried females	-17 (-24 - 6)	-23 (-35 -2)	-7 (-11 - 3)	3 (-7 - 10)	-5 (-17 - 4)	9 (-21-12)	15 (-11 - 3)	12(-6-17)	14 (11-22)	25 (24 - 27)

1:2 (non irradiated cells of control female x irradiated cells of control male), 2:1 (non irradiated cells of control male x irradiated cells of control female) W= wife, H= husband.

Values given in the table are calculated as percent inhibition (%) against the base line measurement and shown as median and interquartile range(IQR),n=10.*P<0.001

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