The Assessment of NK Cytotoxicity and CD56+/CD16+ Phenotype by Flow-cytometry in PBL Isolated from Women with Recurrent Spontaneous Abortion

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ABSTRACT

Background: Human peripheral blood NK cells constitutively express CD56 and CD16 antigens. Peripheral blood NK cells seem to be strongly related with decidual NK cells, and may reflect the decidual NK cell functional status. There are varied reports concerning the relationship between NK cell cytotoxicity in women with recurrent spontaneous abortion. **Objective:** To study NK activity in women with history of RSA by using a non-radioactive cytotoxicity assay. **Methods**: Peripheral blood lymphocytes from RSA and healthy multiparous women were obtained. Lymphocytes were isolated and mixed with K562 NK-sensitive cell line. A non-radioactive method for NK cell cytotoxicity assessment was utilized. Dead K562 cell populations were detected by FACS Calibur flow cytometry, and the data obtained was analysed on cell-Quest software. The proportion of $CD16^+/CD56^+$ cells was then calculated. **Results**: The proportion of NK cells were 9.21% ± 3.06 and 13.48% ± 4.09 in healthy women and RSA, respectively. The percentage of cytotoxicity was determined to be 19.3% ± 3.9 in healthy group and 27.1% ± 6.5 in RSA group with an effector:target ratio of 50:1. The data shows an increase in PBLs potential for in vitro cytotoxicity assay in RSA individuals. The analyses indicate that there is a weak positive correlation between NK cytotoxicity potential and the percentage of NK cells in PBL population. **Conclusion:** The present study demonstrates that the percentage of $CD56^+/CD16^+$ cells increases in individuals with recurrent spontaneous abortion. We conclude that NK cytotoxicity as well as NK number is partially involved in RSA.

Keywords: Flow-cytometry, $CD56^+/CD16^+$, NK Cytotoxicity, Abortion, Peripheral Blood Lymphocytes

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INTRODUCTION

It has been speculated that interaction between integrin molecules on peripheral NK cells and intracellular adhesion molecule-1 on the endothelium of decidual vessels induces the migration of NK cells out of the peripheral blood to the stroma of the endometrium or decidua (1). Although immunophenotypes of the majority of peripheral blood NK cells are different from endometrial NK cells, peripheral blood NK cells seem to be strongly related with decidual NK cells, and may reflect the decidual NK cell functional status. Accordingly, it has been stated that elevated number of peripheral blood NK cells and increased infiltration of endometrial NK cells are related to pregnancy complications (such as preclampsia and miscarriages (8). However, other studies have not proved similar reflection in the uterine NK cell population with peripheral blood NK cells (9).

In addition, there are reports that indicate CD56+/CD16+ lymphocytes in non-pregnant women with recurrent spontaneous abortion of unknown etiology were higher than multiparous pregnant women (5). However, Souya suggested that increased NK activity might not play a role in the occurrence of repeated abortion. Morikawa et al (11) also did not conclude that NK cell activity is the cause of spontaneous abortion (10). Although, many reports published support a powerful role of NK cells in spontaneous abortion, discrepancy still exists.

Gilman et al (12) has concluded that the number of NK cells may not be sufficient in the elevation of NK cell cytotoxicity (12). However, based on many studies which indicate the increased natural killer cell activity in women with recurrent spontaneous abortion, several immunotherapy methods have been designed in order to reduce the NK activation either by using immuno-supresants (13), or immunizing with mononuclear cells (14,15). Others show that women with RSA demonstrate an abnormal cellular immune response by increasing peripheral NK cell and B cells when compared with normal control (6). Therefore, down-regulation of NK cells in women with RSA was associated with a favourable pregnancy outcome. In addition, women with RSA and high NK cell, benefit from IVIG therapy and experience suppression of CD56+ /CD16+ NK cells (16-18). With the use of flow cytometry, it is possible to quantify cytolytic activity on a single cell basis (19). The present study was designed to study the number and the cytotoxicity of NK cells and CD56+/CD16+ phenotypes in peripheral blood lymphocytes (PBL) of women with a history of recurrent spontaneous abortion.

MATERIALS AND METHODS

Patients: Forty-five women with history of three or more RSA were included in our study. Those who had anatomical, genetical, and hormonal abnormalities or infection were excluded after interviewing by a specialist. In addition, forty-five healthy multiparous women with no history of abortion were enrolled in our study as non-RSA control group. The mean age of women with RSA was 29.7 ± 4.5 and for the control group was 31.1±3.8. The mean ± SD of gravidity for control group was 3.3±0.7, and the mean ± SD of spontaneous abortion for RSA group was 3.4 ± 0.7. The PBLs were isolated and used as a source of NK cytotoxicity.

Peripheral blood was collected in EDTA and then peripheral blood lymphocytes (PBL) were separated using a Ficoll-Hypaque gradient technique (Lymphoprep, Norway). Lymphocytes were isolated, washed and brought to a concentration of 5×10^5 cells/ml in RPMI 1640 + 10% FCS (Gibco, Germany). Monocytes were removed by plastic adherence.

The K562 tumor cell line (obtained from Pasteur Institute, Iran) was maintained in a continuous suspension culture in RPMI 1640 + 10% FCS supplemented with L-glutamine, 100 µg/ml streptomycine, and 100U/ml penicillin (Jaber-Ebn-Hayyan, Iran). The cells were maintained in logarithmic growth phase passing them daily. These cells were used as sensitive target cells for the evaluation of natural killer cell cytotoxicity in vitro.

A working solution was prepared by adding 0.5ug/ml of propidium iodide (PI, Sigma) in RPMI 1640+10% FCS. The lymphocytes (as effectors) and K562 (as target) cell lines were mixed and cultured in the same tube with effector:target ratios of 50:1, 25:1 and 12:1, respectively. The non-radioactive assessment method for NK cell cytotoxicity was a modification of the procedure of Vital et al (19) and Gilman-Sachs (12). Briefly, the tubes containing the mixed cells were centrifuged for 3 min at 300g at room temperature, then kept at37°C for 150 minutes in a humified 5% CO2 incubator, and finally the cells were resuspended. In the working solution, a concentration of 1×10^5 cell/ml was prepared to avoid recycling of NK cells. The samples were then incubated for 1 h at 37°C, in 5% CO2, then the cell concentration was brought to 1×10^6 cell/ml prior to flow cytometry. In order to monitor the spontaneous death rate, the only target cells were incubated accompanied with the processing. A final concentration of 1×10^5 cells/ml was running as a control. The cells were analyzed with a FACS Calibur flow cytometer (Becton-Dickinson, USA) using a blue-green excitation light (488nm argon-ion laser). $1x10^4$cells were run through the flow cytometer every time and the data obtained were analysed using a cell Quest software installed in the flow system. The results obtained were illustrated as dot plots or histograms for the cell sub- populations (Fig 1).

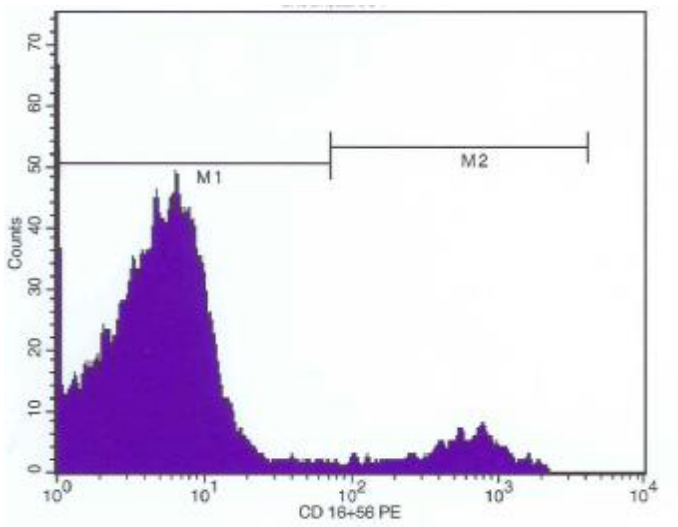

Figure 1. The histogram shows a representative of acquisition data analysis for the proportion of the lymphocytes with the CD16/CD56 PE staining phenotype (M2 population) separated from the population of the lymphocytes gated (M1) based on cell-Quest software on the system.

The incubation time for mixing the effector with target cells for measuring NK cytotoxicity was based on performing several cytotoxicity assays at different time intervals. The data obtained show that the percentage of cytotoxicity increases linearly with the time of incubation, but the percentage of cytotxicity is different from time to time. After 150 minutes of incubation the difference was not significant any more. The kinetics of NK cytotoxicity at different time intervals at a ratio of 50:1 of E:T is summarized in Fig 2.

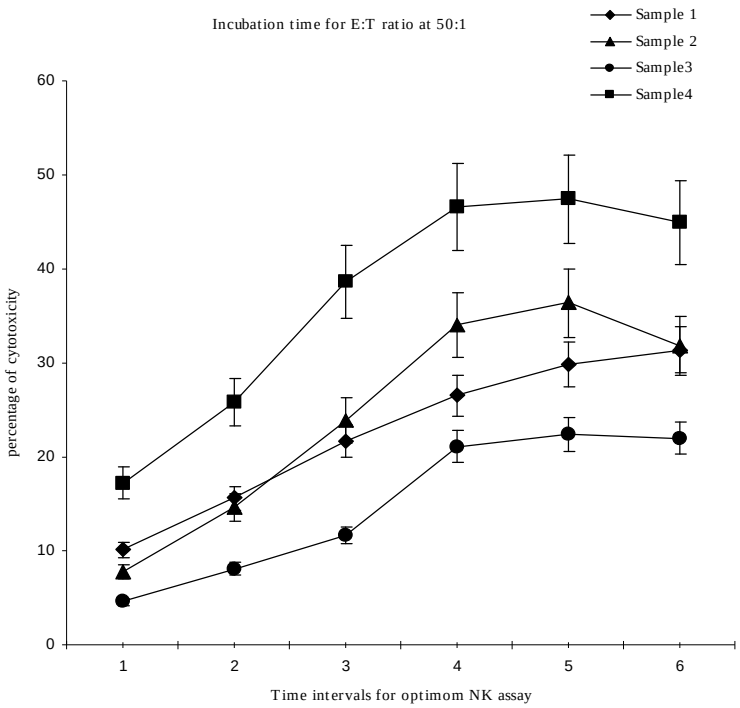

Figure 2. Percentage of NK cytotoxicity at different time intervals and different PBL from RSA & non-RSA women. The graph shows the linearity of NK activity assay at different time intervals. The indicated data is obtained from NK cytotoxicity process in the ratio of 50:1 effector: target cell. The samples with No. 4 and 1 have taken from RSA patients and others from normal healthy group. The number on Y-axis stands for time interval from 60 minutes for 1 to 210 minutes for 6 respectively.

CD16+/CD56+ Phenotyping Assay: PBLs (5×10^5 cells) were suspended in RPMI 1640 medium supplemented with 10% FCS. The cells were stained using phycoerythrin (PE)-anti CD16 monoclonal antibodies (clone B73-1, IgG1) and anti-CD56 (clone MY31, IgG1) (Becton-Dickinson, USA), for 15 min at room temperature in the dark, then washed twice with 3 ml of PBS, and cell suspensions were run in a flow cytometer. Isolated lymphocytes were gated on a forward versus-side scatter histogram. In this histogram, it is possible to gate on the total CD56+/16+ cells from a total cell population for further analysis. The percentage of CD16+/CD56+ cells is shown in a representative histogram (Fig 1).

Statistical Analysis: The data was analyzed using SPSS for windows software installed in IUMS computer network. The Student t-test was used to test statistical significances of the

mean values for ages, NK cells activity and CD56+/CD16+ cell numbers. Correlations were determined by the Pearson test. The level of significance was set at p value < 0.05.

RESULTS

The percentage of CD56+/CD16+ cells in PBLs population was calculated by running 1×10^4 cells in each analysis. The proportion of NK cells was 9.21%±3.06 in healthy women, and 13.48%±4.09 in RSA women. When the means were compared, the statistics shows significant differences, with P value of 0.04.

The results of NK cytotoxicity percentage in non-RSA group and RSA group are indicated in Table 1. The percentage of cytotoxicity was 19.3%± 3.9 for the E:T ratio of 50:1, 13.97%± 3.5 for E:T ratio of 25:1 and 8.87±2.1 for E:T ratio of 12:1, respectively.

The data obtained for RSA group was 27.1%±6.5 for E:T ratio of 50:1, 21.34±6.3 for E:T ratio of 25:1 and 14.5±4.2 for E:T ratio of 12:1, respectively. Statistical analysis shows the differences between the mean of cytotoxicity in non-RSA group and RSA group for all the ratios to be significant (P=0.000). The data demonstrates an increase in PBLs potential for in vitro cytotoxicity assay in RSA individuals.

Table 1. In vitro NK cytotoxicity measurements by flowcytometry

E:T ratio	RSA	Non-RSA	P value
50:1	27.1±6.5	19.3±3.9	0.000
25:1	21.4±6.3	13.97±3.5	0.000
12:1	14.5±4.2	8.87±2.1	0.000

PBL were the source of NK cells for both RSA and non-RSA women. The tests were performed 2-3 times for each sample and mean ± SD for each ratio tested are presented. The P values show that the differences of means between the two groups are significant.

The analyses indicate that there is a weak positive correlation between NK cytotoxicity potential and the percentage of NK cells in PBLs population. The Pearson correlation for RSA group was r=0.354, with P=0.017 (for E:T = 50:1) and r=0.366, with P=0.013 (for E:T = 25:1) and r=0.264 with P=0.080 (for E:T =12:1). Moreover, the same analysis performed for the normal control group shows r = 0.339, with P=0.023 (for E:T = 50:1), and r=0.281, with P=0.062 (for E:T = 25:1), and r=0.271 with P=0.072 (for E:T =12:1). Therefore, it seems that the correlation between NK cytotoxicity and the NK numbers in RSA individuals is much higher. However, this correlation for non-RSA group is not statistically significant. In addition, the correlation between the age of the women with RSA and/or the age of women in control group with NK cytotoxicity was not statistically significant.

DISCUSSION

In the present study NK cell cytotoxicity and the percentage of peripheral blood CD56+/CD16+ cells in both normal controls and those with RSA were investigated using flowcytometry. Consistent with the data obtained by Gilman et al (12) our study, indicates that flowcytometry is a reproducible method for evaluating the NK cytotoxicity compared with Cr51 release method (12).

Several recent studies have reported significant changes in circulating immunocompetent cells of women with RSA (7,9,10). Our results demonstrated that in women with a

history of RSA, the NK cytotoxicity were significantly higher than the control group. Our present finding is in agreement with some previous studies indicating that in RSA patients NK cells cytotoxicity potential against the NK sensitive target cells (K562) are significantly higher than normal multiparous women (7,12,21). However, Morikawa et al (10) concluded that neither NK cell activity nor subsets could make a significant difference in relation to the cause of spontaneous abortion (10). In addition, Souza et al (11) have shown that NK activity using 51Cr releasing assay, did not differ between these two groups when expressed as specific cytotoxicity, and reduced in patients with RSA when expressed as lytic unit (11).

At prersent, the contradictory results could be explained by disadvantages of the 51Cr releasing assay (11,22). However, in recent years, alternative approaches have been proposed using fluorescent dyes and flow cytometry for analysing cytotoxicity. Flow cytometry provides a powerful approach to the measurement of cell death and cell killing in a cell population (19,23).

The present study has also demonstrated that the percentage of CD56+/CD16+ cells increase in RSA group. This finding is in consistent with the observations by others such as Kwak et al and Emmer et al (6,21,24). However Souza and Morikawa reports do not support this finding(10;11).

Our results, also, show that there is a weak relationship between the number of CD56+/CD16+ population and the percentages of NK cytotoxicity in RSA group (for at least two ratios), but not for all the ratios in control group. The difference could indicate that the same number of NK cells may display variable cytotoxic activity. This observation however, is not in agreement with that of Gilman-Sachs et al (12) who report a lack of correlation between NK cell cytotoxicity and the quantity of CD56+/CD16+ cell population (12). Whiteside et al has also observed no relationship between NK cell phenotype and function in RSA women (25). On the other hand, other studies have reported a strong correlation between NK cell number and cytotoxicity (5). It should be noted that most of these studies used 51Cr-release assay.

According to previous studies, the possible explanation for this discrepancy may be as follows: CD56+/CD16+ effector cells may have varying amounts of perforin and granzymes, the granules that cause the lysis of target cells. Therefore, some NK cells could have an extra ability for killing (12).

There are several reports that show the efficacy of applied immunotherapy or immunosuppression based on the down-regulation of NK cell activity in high risk pregnant women with a history of RSA in favour of pregnancy outcome (17;26). Although in the present study, we did not consider the cytokine TH1/TH2 ratios, some reports show their importance in pregnancy outcome(3,27). Our results indicate that NK cytotoxicity as well as NK cell number are partially involved in RSA. Other immunological aspects such as cytokines interference, the frequency of PBMC subsets, and NK receptor expression might be considered to explain recurrent spontaneous abortion.

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REFRENCES

1. Kammerer U, Marzusch K, Krober S, Ruck P, Handgretinger R, Dietl J. A Subset of CD56+ Large Granular Lymphocytes in First-Trimester Human Decidua Are Proliferating Cells. Fertil Steril. 1999;71:74-9.
2. Lanier LL. NK Cell Receptors. Annu Rev Immunol. 1998;16:359-93.
3. Mendes R, Bromelow KV, Westby M, Galea-Lauri J, Smith IE, O'Brien ME, et al. Flow Cytometric Visualisation of Cytokine Production by CD3-CD56+ NK Cells and CD3+CD56+ NK-T Cells in Whole Blood. Cytometry. 2000 Jan 1;39(1):72-8.
4. Chuntharapai A, Lee J, Hebert CA, Kim KJ. Monoclonal Antibodies Detect Different Distribution Patterns of IL-8 Receptor A and IL-8 Receptor B on Human Peripheral Blood Leukocytes. J Immunol. 1994;153:5682-8.
5. Beer AE, Kwak JY, Ruiz JE. Immunophenotypic Profiles of Peripheral Blood Lymphocytes in Women With Recurrent Pregnancy Losses and in Infertile Women With Multiple Failed in Vitro Fertilization Cycles. Am J Reprod Immunol. 1996;35:376-82.
6. Kwak JY, Beaman KD, Gilman-Sachs A, Ruiz JE, Schewitz D, Beer AE. Up-Regulated Expression of CD56+, CD56+/CD16+, and CD19+ Cells in Peripheral Blood Lymphocytes in Pregnant Women With Recurrent Pregnancy Losses. Am J Reprod Immunol. 1995;34:93-9.
7. Kwak JY, Gilman-Sachs A, Moretti M, Beaman KD, Beer AE. Natural Killer Cell Cytotoxicity and Paternal Lymphocyte Immunization in Women With Recurrent Spontaneous Abortions. Am J Reprod Immunol. 1998;40:352-8.
8. Quenby S, Bates M, Doig T, Brewster J, Lewis-Jones DI, Johnson PM, et al. Pre-Implantation Endometrial Leukocytes in Women With Recurrent Miscarriage. Hum Reprod. 1999;14:2386-91.
9. Ntrivalas EI, Kwak-Kim JY, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, et al. Status of Peripheral Blood Natural Killer Cells in Women With Recurrent Spontaneous Abortions and Infertility of Unknown Aetiology. Hum Reprod. 2001 May;16(5):855-61.
10. Morikawa M, Yamada H, Kato EH, Shimada S, Ebina Y, Yamada T, et al. NK Cell Activity and Subsets in Women With a History of Spontaneous Abortion. Cause, Number of Abortions, and Subsequent Pregnancy Outcome. Gynecol Obstet Invest. 2001;52:163-7.
11. Souza SS, Ferriani RA, Santos CM, Voltarelli JC. Immunological Evaluation of Patients With Recurrent Abortion. J Reprod Immunol. 2002;56:111-21.
12. Gilman-Sachs A, DuChateau BK, Aslakson CJ, Wohlgemuth GP, Kwak JY, Beer AE, et al. Natural Killer (NK) Cell Subsets and NK Cell Cytotoxicity in Women With Histories of Recurrent Spontaneous Abortions. Am J Reprod Immunol. 1999;41:99-105.
13. Rigal D, Vermot-Desroches C, Heitz S, Bernaud J, Alfonsi F, Monier JC. Effects of intravenous immunoglobulins (IVIG) on peripheral blood B, NK, and T cell subpopulations in women with recurrent spontaneous abortions: specific effects on LFA-1 and CD56 molecules. Clin Immunol Immunopathol. 1997;71:309-14.
14. Higuchi K, Aoki K, Kimbara T, Hosoi N, Yamamoto T, and Okada H. Suppression of Natural Killer Cell Activity by Monocytes Following Immunotherapy for Recurrent Spontaneous Aborters. Am J Reprod Immunol. 1995;33:221-7.
15. Jerzak M, Rechberger T, Baranowski W, Semczuk M. Immunotherapy As an Effective Treatment of Recurrent Spontaneous Abortion--Own Experience Ginekol Pol. 2003;74:1107-11.
16. Kwak JY, Kwak FM, Ainbinder SW, Ruiz AM, Beer AE.Elevated Peripheral Blood Natural Killer Cells Are Effectively Downregulated by Immunoglobulin G Infusion in Women With Recurrent Spontaneous Abortions. Am J Reprod Immunol. 1996;35:363-9.
17. Konova E, Ivanova I, Petrova P, Popov I, Andreeva Kh, Lukanov Ts. Effect of Intravenous Gamma-Globulin Therapy on Lymphocyte Population in Pregnant Women With Antiphospholipid Antibodies.Akush Ginekol (Sofiia). 2004;43:3-10.
18. Kwak JY, Kwak FM, Gilman-Sachs A, Beaman KD, Cho DD, Beer AE. Immunoglobulin G Infusion Treatment for Women With Recurrent Spontaneous Abortions and Elevated CD56+ Natural Killer Cells. Early Pregnancy. 2000;4:154-64.
19. Vitale M, Neri LM, Comani S, Falcieri E, Rizzoli R, Rana R, et al. Natural Killer Function in Flow Cytometry. II. Evaluation of NK Lytic Activity by Means of Target Cell Morphological Changes Detected by Right Angle Light Scatter. J Immunol Methods. 1989;121:115-20.
20. Lozzio CB, Lozzio BB. Human Chronic Myelogenous Leukemia Cell-Line With Positive Philadelphia Chromosome. Blood 1975;45:321-34.
21. Emmer PM, Nelen WL , Steegers EA, Hendriks JC, Veerhoek M, Joosten I. Peripheral Natural Killer Cytotoxicity and CD56(Pos)CD16(Pos) Cells Increase During Early Pregnancy in Women With a History of Recurrent Spontaneous Abortion. Hum Reprod. 2000;15:1163-9.
22. Souza SS, Castro FA, Mendonca HC, Palma PV, Morais FR, Ferriani RA, et al. Influence of Menstrual Cycle on NK Activity. J Reprod Immunol. 2001;50:151-9.
23. King MA. Detection of Dead Cells and Measurement of Cell Killing by Flow Cytometry. J Immunol Methods. 2000;243:155-66.
24. Thum MY, Bhaskaran S, Abdalla HI, Ford B, Sumar N, Shehata H, et al An Increase in the Absolute Count of CD56dimCD16+CD69+ NK Cells in the Peripheral Blood Is Associated With a Poorer IVF Treatment and Pregnancy Outcome. Hum Reprod. 2004;19:2395-400
25. Whiteside TL, Bryant J, Day R, Herberman RB. Natural Killer Cytotoxicity in the Diagnosis of Immune Dysfunction: Criteria for a Reproducible Assay. J Clin Lab Anal. 1990;4:102-14.
26. Shakhar K, Ben-Eliyahu S, Loewenthal R, Rosenne E, Carp H. Differences in Number and Activity of Peripheral Natural Killer Cells in Primary Versus Secondary Recurrent Miscarriage. Fertil Steril. 2003;80:368-75.
27. Lidstrom C, Matthiesen L, Berg G, Sharma S, Ernerudh J, Ekerfelt C.Cytokine Secretion Patterns of NK Cells and Macrophages in Early Human Pregnancy Decidua and Blood: Implications for Suppressor Macrophages in Decidua. Am J Reprod Immunol. 2003;50:444-52.
28. Matsubayashi H, Hosaka T, Sugiyama Y, Suzuki T, Arai T, Kondo A, et al. Increased Natural Killer-Cell Activity Is Associated With Infertile Women. Am J Reprod Immunol. 2001;46:318-22.