Different Immune Responses in Dangerous O Blood Donors

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ABSTRACT

Background & Objective: Dangerous O is very important to transfusion medicine and there has been reports by Food and Drug Administration (FDA) regarding some death relating incidences. As high iso-antibody production is closely associated with different immune reactions, a survey on the different immune response of dangerous O donors can lead to understanding their immune response profile. Objectives were to assess different immune responses in dangerous O cases.

Methods: Two groups of donors were selected after performing titration as a high titer (>512) and non-high titer (<128). Then CBC, CD markers, total immunoglobulin, complement assay, anti-VZV, -CMV, -EBV, -HSV, -Rubella, -*Toxoplasma gondii*, -HBV, -ASO, total protein and albumin, protein electrophoresis, lymphocyte proliferation, and gene expression of INF-gamma, IL2/4/10 were evaluated on both study groups.

Results & Conclusion: Total IgG, IgM, and IgA was higher in high titer group. Moreover, after using PHA and LPS, gamma globulins and lymphocyte proliferation were significantly higher in high titer cases. Real-time PCR also showed higher IL-2 production in high titer group. Identification of high responder's characteristics can be efficient in many complications. Moreover, high titer donors are dangerous for transfusion medicine. This pilot study showed differences in immune responses between HR and LR O blood donors for the first time. So, other aspects of the immune system such as genetic differences can be surveyed.

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Introduction

Dangerous O has been a critical issue in transfusion medicine which is referred to individuals with O blood type who carry high levels of iso-antibodies that can destroy red blood cells of recipients (1). Food and Drug Administration (FDA) has also reported some incidences of death following transfusion of blood products from dangerous O cases (2). Large amounts of anti-A and anti-B can cause severe hemolytic transfusion reaction (HTR) in an incompatible recipient. (3) The exact cause of dangerous O phenomenon hasn't been explained; however, a combination of genetic and environmental factors are very probable candidates (4). Survey on immunological responses can bring valuable information about different immune responses in dangerous O donors.

Considering the strong ability of the dangerous O's immune system in responding to polysaccharide antigens of blood group system, evaluation of both humoral and cellular immune responses against many other antigens (carbohydrates or proteins) may reveal different immune characteristics in dangerous O donors. Besides, antibodies are the most important part of the humoral immunity and have close contribution to other parts of the immune system especially cellular immunity (5). Therefore, they provide a valuable paradigm for studying different aspects of the immune responses.

All individuals do not react similarly against a common antigen. To be a high or low responder (LR) to an antigen is highly associated with genetic factors and the ability of the immune system to select a specific major histocompatibilitycomplex (MHC) and Ig dependent repertoire.

In this pilot study, for the first time, we evaluated different immune responses in dangerous O cases and aimed to assess different immune responses in high titer cases. Hence, different immunologic assays targeting humoral, cellular, and innate immunity were designed.

Materials and Methods

Ethical Issues

This study was approved by the ethics committee in the high institute for research and education in transfusion medicine, Iranian Blood Transfusion Organization. All of the donors filled out a form about their background and signed a consensus letter and were informed about the process of the work.

Subject Studied

Around 400 blood donors with O blood type were selected randomly, at the Iranian Blood Transfusion Organization from November 2015 to February 2017. After two-fold serial dilution, the final titers of anti-A and anti-B were recorded (from two to 4096) for all samples. Two groups of non-High Titer (the lowest titer results, <128, n=57) and High Titer (the highest titer results, >512, n=28) were asked to donate blood again. As only some of them were available at the time of the study, two groups (High titer and non-high titer) of donors with 11 members each (totally 22) were gathered. Then the following tests were applied to their samples.

Complete Blood Count

The complete blood count test was performed on fresh whole blood and the number of neutrophils, basophils, lymphocytes, monocytes, and eosinophils were evaluated in each sample by XT-2000i, Sysmex Company, Sweden.

Lymphocyte's Markers by Flow Cytometry

CD3-FITC/HLA-DR-PE and CD56-PE from BD Biosciences and also CD20-FITC, CD4-FITC, CD8-PE, CD16-FITC, CD57-FITC, CD45-RO-PE, CD5-FITC, and CD19-PE from Dako Corporation were exploited by CyFlow Space-Sysmex Partec, Sysmex Company, Sweden. Direct conjugation of all of those antibodies was used. Murine anti-human IgG1 is also used as specific isotype control (x0932: Dako and 349526 BD: Bioscience, USA).

Immunoglobulin and Complement Levels

Measuring the levels of serum proteins including IgG, IgM, IgA, C3, and C4 was performed through immunodiffusion technique in a semi-solid media according to the Baharafshan Kit, Iran. In addition, Total IgE level was measured according to ELISA kit (OMEGA, product code: GD09, UK) and the results were read at 450nm.

CH50 Assay

This was performed through single radioimmuno diffusion (SRID) assay according to the Baharafshan kit, Iran.

Antibody Production Against Common Infections

Anti-VZV, -CMV, -EBV, -HSV, -Rubella, -*Toxoplasma gondii*, (IgM and IgG) were measured using Trinity Biotech ELISA kit (Ireland) for all of the cases. Anti-HBV was measured by Enzygnost anti-HBs-II kit, Germany.

Anti-Streptolysin O

ASO detection was performed using OMEGA kit and rapid method.

Total Proteins

Measurements of grams of protein per 100 grams of sample was performed based on the intensity of the violet color after reaction of some protein's peptide with Cu^{2+} in Biuret reagent. It was read at 540nm.

Albumin assay

Albumin, is able to form a colored complex with Bromocresol Green (BCG). The intensity of the colored complex was used as indicative for the concentration of albumin and was detected by a spectrophotometer.

Cellulose Acetate Electrophoresis

Using TITAN III cellulose acetate sheets (60*67), proteins were separated based on their electric charge and molecular weight (Junior 24 Helena scanner).

Cell Division with CSFE

Buffy coat was isolated from each sample (by Ficol; GE Healthcare Ficoll-Paque™ PLUS, Denmark) and optimum amounts of phytohemagglutinin (PHA; 10576,015 ،Invitrogen, USA) and lipopolysaccharide (LPS; Sigma, 0111, Lipopolysaccharides from Escherichia coli: B4 ، L2630-10MG, United Kingdom) were used as mitogens.

To set up the test, several concentrations of LPS including 100, 50, 25, 12.5 and 7.25 µg/mL-1 of LPS were used for 400000, 200000, 100000, and 50000 cells in 24, 48, and 72h incubation time. For PHA, one, two, four, eight, and 10 µL of PHA were used for 400000, 200000, 100000, and 50000 cells in 24, 48, and 72h incubation time.

Cell division rate was surveyed using carboxyfluorescein succinimidyl ester (CFSE, Life Technologies, Invitrogen, Catalog numb: C34554, USA). Fluorescent emitted from each sample measured by Flowcytometry technique. Accordingly, the best amount of PHA was reached by using two µL of PHA (1.5 %) for 200000 cells at 72h. The optimum concentration of LPS was obtained as 50 µg/mL-1 of LPS for 2000000 cells at 72h for the test.

Real-Time PCR

INF-gamma, IL2/4/10 were evaluated by Real-Time PCR technique for all 22 samples before and after using PHA and LPS as mentioned above.

RNA extraction was performed through mechanical homogenization of whole blood samples using QIAzol Lysis Reagent. In following, RNA clot was extracted by chloroform, Isopropanol, and 75% ethanol sequentially. Finally, RNA quality and purity was monitored using 260/280 and 260/230 absorbance rate by Nano-drop 1000. cDNA synthesis

Table 1. Mean and standard deviation of $2^{-\Delta\Delta CT}$ amounts in high titer and non-high titer group for INF-gamma, IL-2, IL-4, and IL-10. P-value also showed the result of the comparison between two groups

		High Titer		Non-High Titer		P-Value
		Mean**	Std.Deviation	Mean**	Std.Deviation	
IL-2	PHA	12.53	30.53	4.532	10.29	0.03
	LPS	68.69	121.1	1.33	1.369	0.02
IL-4	PHA	2.075	2.834	1.885	4.752	0.89
	LPS	20.62	36.7	2.005	1.749	0.83
IL-10	PHA	24.69	25.31	9.453	18.96	0.18
	LPS	13.49	20.53	8.986	11.08	0.83
INF-γ	PHA	239.9	581.8	374.3	820.4	0.68
	LPS	3.589	5.232	8.608	22.36	0.47

**: $2^{-\Delta\Delta CT}$
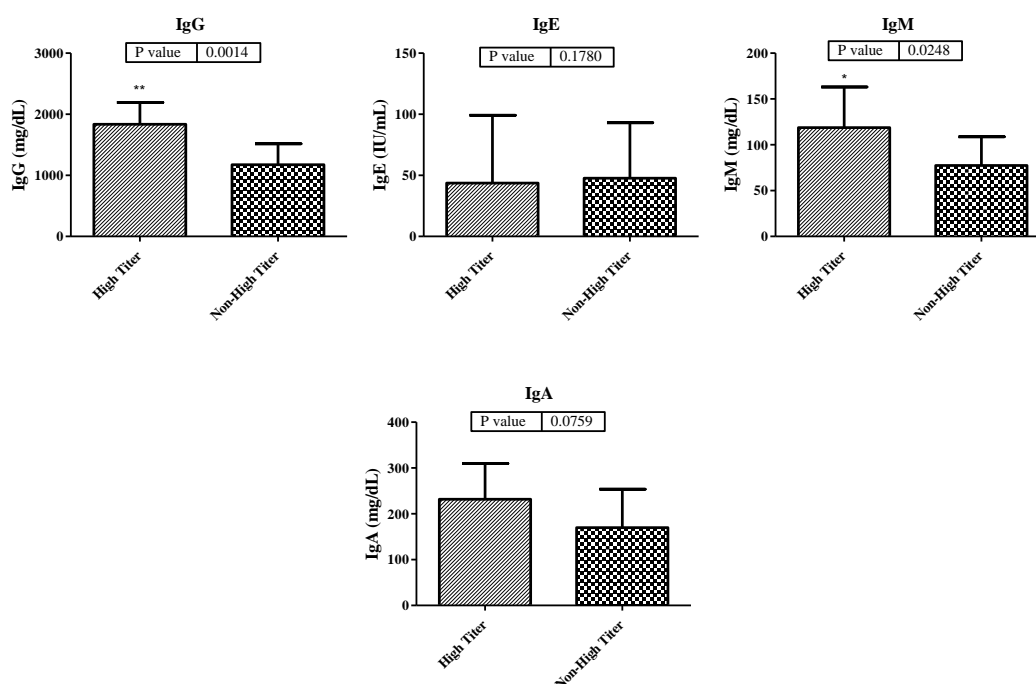
Fig. 1. Levels of four immunoglobulin classes in high titer and non-high titer groups. Comparison of four Ig levels showed that total IgG and total IgM were meaningfully higher in high titer group.

was also performed based on the Quantitect Reverse Transcription Kit from QIAgen.

The cDNA samples were used for real-time PCR technique and CT amount for each sample (before and after using mitogen) was obtained. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was the housekeeping gene for internal control. After 45 cycles, ΔCT control, ΔCT sample, ΔΔCT, and $2^{-\Delta\Delta CT}$ were measured for all cytokines and compared between two groups.

Statistical Analysis

Comparison between the two groups (High titer and non-High titer) and data analysis were performed in Graphpad Prism (GraphPad Software, San Diego, CA 92108), version 5.04 using T-test (Mann-Whitney). A P-value less than 0.05 were considered statistically significant.
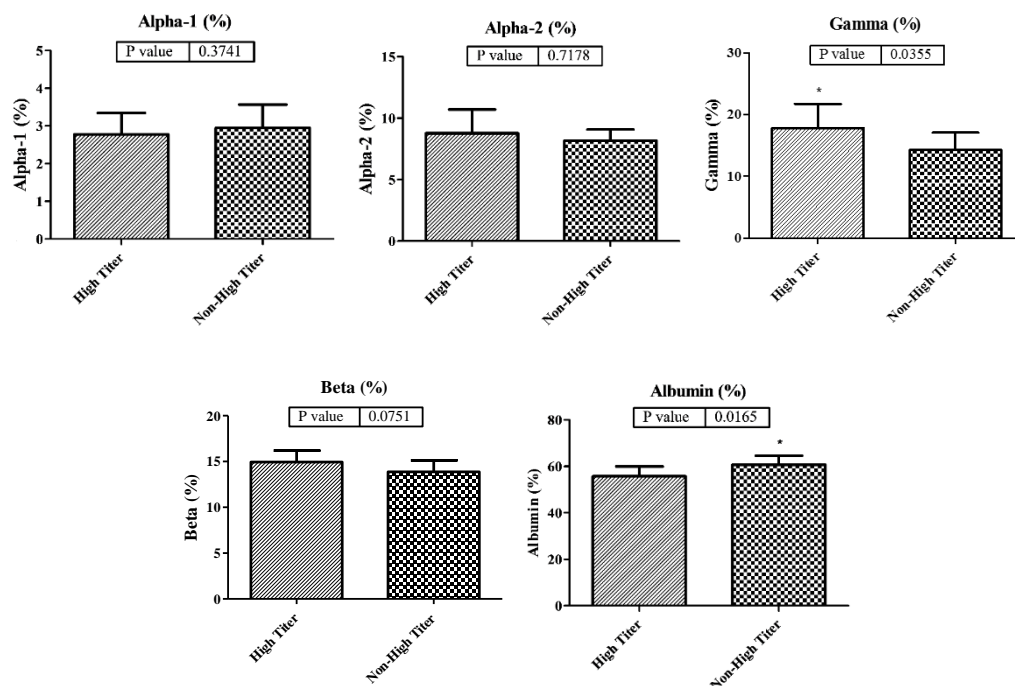
Fig. 2. Comparison of the levels of γ, β, α2, α1, and albumin in high titer and non-high titer group. Comparison shows significant differences between the two groups.

Results & Discussion

CBC

Comparison between high titer and non-high titer group showed no significant differences in number of Lymphocytes, Neutrophils, Basophils, Monocytes, Eosinophils, and WBCs (P-values = 0.26, 0.43, 0.94, 0.32, 0.57, 0.84 respectively).

Lymphocyte CD markers

Percent of CD5, CD19, CD20, and co-expression of CD5 and CD19 was determined for B cells (P-values= 0.84, 0.84, 0.97, 0.86 respectively). CD4, CD8, CD3, CD4/CD8, HLA-DR, and co-expression of CD3 and HLA-DR were analyzed for T cells (P-values =0.64, 0.59, 0.89, 0.71, 0.97, 0.44 respectively). CD16, CD56, and CD45RO were also examined for Nk cells and memory cells respectively (P-values =0.84, 0.26, 0.21 respectively). Considering P-value<0.05, none of them showed significant differences between groups.

Total Immunoglobulins, Complement Components, and CH50

Significant difference was seen in levels of IgG and IgM so that both were higher in high titer group (P-value: IgG=0.00 and P-value: IgM=0.02). IgA was also higher in high titer group; however, the difference was not statistically significant (P-value: IgA=0.07), (Figure 1). Statistical analysis of, C3, C4, and CH50 didn't show any meaningful difference (P-values =0.46, 0.62, 0.23 respectively).

Antibody Production Against Common Pathogens

Two groups didn't show any significant differences in CMV (P-value; IgM=0.97/ IgG=0.79), EBV (P-value; IgM=0.46/ IgG=0.89), HSV (P-value;

IgM=1.00/ IgG=0.92), Rubella (P-value; IgM=0.31/ IgG=0.84), Toxo (P-value; IgM: 0.31/ IgG=0.23), VZV (P-value; IgM=0.09/ IgG=0.08), and anti-HBs (P-value=0.83).

ASO

ASO test showed no meaningful difference between two groups (P-value=0.36).

Total Proteins and Albumin Assay

Total protein levels was not significantly different between the two groups. However, P-value of 0.0059 showed higher albumin levels in non-high titers group.

Proteins Electrophoresis

α2, α1 analysis represented no significant difference between groups. γ and β globulins were higher in high titer group. However, only γ-globulins (%) difference was statistically meaningful (P-value=0.03). Besides, albumin levels were significantly lower in high titer group (P-value=0.01). Comparison of γ, β, α2, α1, and albumin (%) is shown in Figure 2.

Cell Division with CSFE

Cell division rates are represented in Figure 3 for one sample of each group under the influence of PHA, LPS, and the control sample. Using both PHA and LPS, lymphocyte proliferation was significantly higher in high titer group (Figure 4).

Real-Time PCR

For each of the cytokine, ΔCT (before using PHA and LPS), ΔCT (after using mitogens), ΔΔCT, and $2^{-\Delta\Delta CT}$ were measured and compared between two

groups. Mean, standard deviation and P-value for the final results of each cytokine are revealed in Table 1. The results exhibited that IL-2 gene expression was significantly higher in high titer group after using PHA and LPS (P-value; IL2/LPS=0.02 and IL2/PHA=0.03).

In present study, we tried to assess immune responses using the most practical techniques. The immune system of all blood recipients do not react similarly. Some of them are high responders (HR) and some of them are medium or low responders (LR) (6, 7). Different Immune response (Ir) genes control high or low immune response against various epitopes of an antigen (8-10).

B cell assessment was performed to see if the high titer group has a different immune response profile. In our study all markers of B cell were in normal range and no association was found between dangerous O and B cell markers.

According to Biozzi *et al.*, serum Igs level before and after sensitization were significantly different between HRs and LRs which was mainly associated with IgG levels (11). IgM and IgA represented a lower difference between the two groups. In our study, IgG and IgM levels were meaningfully higher in high titer group. We also previously showed IgG2 levels were higher in high titer group (Accepted for publication, Arabi, S). It has been shown that IgG2, IgG3, and IgG4 are produced at higher levels in high responders (12). Total immunoglobulin levels and B cell ability in producing functional antibodies are good criteria for assessment of B cell function (13). We showed total IgG and IgM were significantly higher in high titer group. Although, IgA was also insignificantly higher in this group in accord to the study of Biozzi *et al.* (11).

Higher gamma and beta globulin percent in our study showed higher levels of Igs in high titer group. Therefore, dangerous O can be associated with higher Ig production. Lower levels of albumin in the non-high titer group in our data revealed the equal total protein level in both groups.

High iso-antibody production could be due to the higher immune stimulation and cytokine production against a common microbial structure (14). However, our study showed no different antibody production against common microbial infections in our society, including CMV, EBV, HSV, Rubella Virus, *T. gondii*, and HBV and ASO which determined no association with the higher immune response against A and B antigens. On the other hand, some studies showed that pneumococcal preparation or influenza viruses shared A-like substance which is able to instigate the immune system and cause high titer anti-A in dangerous O donors (15).

Lymphocyte proliferation assay (LPA) using PHA and LPS is usually used to determine the intensity of the immune response in individuals with different immune profiles (16, 17). Our results revealed increased proliferation levels against PHA and LPS in dangerous O lymphocytes. In addition, we showed

PHA stimulated more proliferation than LPS. It may be due to the secretion of increased levels of pro-inflammatory cytokines. Studies on hepatitis B virus showed PHA highly promotes cytokine production in HRs which is due to the more pro-inflammatory cytokines (18). In general, LPS initiates a severe inflammatory response in WBCs that could be different from one person to another.

Increased CD3+ and CD4+ cells can be the sign of a shift from Th1 into Th2 responses (19). Moreover, increased levels of CD4+CD45RO+ cells is associated with high levels of INF-gamma production (20). Evaluation of T cell markers in our study didn't show any correlation with dangerous O phenomenon.

Evaluation of cytokines can delineate the quality of an immune response (21, 22) . Th1 cells act mainly by producing IL-2 and INF-gamma and decreased levels of IL-2 and INF-gamma is associated with a shift from Th1 to Th2 responses (23). We used IL-2 and INF-gamma as main cytokines of T cell activation and IL-4 and IL-10 for Th2 assessment.

Pro-inflammatory cytokines have been shown intensified in HRs which is probably associated with the ability of the immune system to act more effectively against bacterial element like LPS (24). Moreover, higher IL-2 gene expression in dangerous O donors showed that it probably plays a role in the higher immune response of dangerous O cases.

Identification of high responder's characteristics and genetic features can be efficient for many complications to be prevented. For example in a patient with thalassemia or sickle cell anemia who received high amounts of blood, there is a risk or high alloantibody production (25). Therefore, defining a high responder profile may lead to use of compatible blood products for them. Moreover, genetic identification to predict responder/non-responder profile to the RhD antigen was also performed by Tan JC and colleagues at Australian Red Cross Blood Service (26). They found responder profiles of anti-D donors and proposed this could potentially be used to new donors and transfusion-dependent patients. Accordingly, High responder plasma would be a good choice to be collected and used to manufacture antibody products such as anti-D immunoglobulin. This is a pilot study which evaluated immune responses in dangerous O blood donors for the first time. Our results cannot confirm any exact characteristics as criteria to distinguish dangerous O donors but they can show there are some differences between two groups that can be used as the basis for further study in future especially genetic assessment that can help personalized medicine.

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Conflict of Interest

The authors declared that there is no conflict of interest regarding the publication of this article.

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