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ORIGINAL ARTICLE

Study of the Serum Immunoglobulin and Cell-Mediated Immunity in Patients with Congenital Severe Hemophilia

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SUMMARY

Background: **It has been shown that a close relationship exists between the immune system and coagulation cascade. Hemophilia A is an X-linked, recessive bleeding disorder caused by deficiency of functional plasma clotting factor VIII that is classically treated with factor VIII replacement therapy. Despite this, some patients produce inhibitors or antibodies against epitopes of infused factor VIII, indicating the activation of the adaptive immune system. The aim of this study was to evaluate the change in the T cell frequency and serum immunoglobulin level in patients with congenital hemophilia A, especially those who produce inhibitors against factor VIII.**

Methods: **This is a cross-sectional, case-control study on congenital hemophilia A patients with severe factor VIII deficiency. Twenty-eight hemophilia A male patients were randomly selected along with twenty age-matched healthy males, as the control group. Serum immunoglobulin concentration was measured by nephelometry (for IgG, IgA and IgM) and enzyme-linked immunosorbent assay (ELISA) (for IgE) and the frequency of $CD4^+$ and $CD8^+$ T cells were calculated using a flow cytometry method.**

Results: **Serum IgG was significantly higher in hemophilic patients compared to controls (14.35 ± 3.60, vs. 12.4 ± 1.72, p = 0.014). Among IgG subtypes, the IgG_1 antibody was significantly higher in hemophilia patients than control group ($p < 0.001$). The frequency of $CD4^+$ as well as $CD8^+$ T cells did not significantly differ between patients and control group ($p > 0.05$). There was no significant difference between patients with and without inhibitors regarding serum immunoglobulin level, different IgG subtypes, the frequency of CD4+ and CD8+ T cells as well as CD4/CD8 ratio ($p > 0.05$).**

Conclusions: **Patients with hemophilia A have high levels of serum immunoglobulin especially IgG_1. Therefore, further larger studies along with close observation and evaluation of the presence of serum inhibitor is recommended every 3 months.**

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INTRODUCTION

The coagulation system is characterized by a consecutive series of events culminating in the production of thrombin and subsequent conversion of fibrinogen into a fibrin clot. Interestingly, it has been shown that a close

relationship exists between the immune system and coagulation cascade [1-4]. Immune-mediated inflammation may lead to activation of the coagulation cascade, and conversely, coagulation can affect inflammatory activity, both are implicated in maintaining homeostasis [1-4].

Hemophilia A (HA) is an X-linked, recessive bleeding disorder caused by a deficiency of functional plasma clotting factor VIII (FVIII), which may be inherited or arise from spontaneous mutation with an incidence of one in 5,000 to 30,000 males worldwide. Depending on level and type of factor deficiency, patients may present without any symptoms, or with easy bruising even in mild injury, and in the case of severe deficiency with spontaneous hemorrhage [5,6].

The conventional treatment for hemophilia A involves intravenous administration of exogenous FVIII. However, some patients (about 20 - 30%) develop inhibitor which is the most serious complication after replacement therapy [7-9]. Inhibitors are neutralizing antibodies (mainly IgG_1 and IgG_4 subclasses) directed against the administered FVIII that reduces its effectiveness [7-9]. The main reason for the production of these antibodies is that the exogenous factor is known as "foreign protein" for the host immune system which can elicit activation of the adaptive immune response [9].

As a result, these non-self-antigens are recognized by specific T cells leading to T cell activation, cytokine production that stimulates B cell activation and plasma cell differentiation and finally, production of specific antibodies against plasma FVIII [9,10].

Little is known about the status of the adaptive immunity in hemophilia patients especially those who produced inhibitors to FVIII. Therefore, in this study, we evaluated the frequency of T cell subsets and serum immunoglobulin (Ig) level in HA patients compared to healthy controls as well as in those patients who developed inhibitors compared to inhibitor negative ones.

MATERIALS AND METHODS

Study design and patient selection

This is a cross-sectional, case-control study on HA male patients with severe factor deficiency, who have been registered at the Comprehensive Hemophilia Center, affiliated to Shiraz University of Medical Sciences from January to December 2017.

We enrolled 28 severe HA male patients (16 - 48 years old), who were randomly selected from 220 severe hemophilia patients. Inclusion criteria were severe type of the disease (factor level below 1%), negative history of collagen vascular or chronic infectious diseases, negative serologic result for hepatitis B or C within the past two months, and negative history of any immunosuppressive drugs consumption like glucocorticosteroids and cyclosporine. Exclusion criteria were any viral or bacterial infectious diseases during the week before sampling, positive history of hospital admission due to acute infectious diseases in last two weeks, and confirmed autoimmune, inflammatory or allergic diseases. All patients received plasma factor concentrates on-demand and had no previous history of immune tolerance induction. Twenty healthy age-matched males were randomly selected and considered as the control group.

The study was approved by the institutional ethics committee and all enrolled patients and/or guardians signed a written informed consent.

Evaluation of the serum immunoglobulin levels

Six milliliters of peripheral blood were obtained from participants, which was equally divided in EDTA-K_2 and clot activator tubes (KIMA, Italy). Total immunoglobulin A, M, and G levels were measured by nephelometry method using the MININEPH Plus instrument (Banding Site Company, England), whereas the level of IgE was measured by ELISA method, Cobas 6000 analyzer series (Roche/Hitachi, Germany). The IgG subtypes including IgG_1, IgG_2, IgG_3, and IgG_4 were also measured.

Flow cytometric analysis of T cell subsets

Three milliliters of peripheral blood were obtained from all participants in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Then, peripheral blood mononuclear cells (PBMCs) were isolated from each individual using Ficoll-hypaque density gradient centrifugation (Inno-train, Germany). Cells were washed twice with phosphate buffered saline (PBS) (1X) prior to flow cytometry analysis and were adjusted to 5×10^5 cells/ 100 µL staining buffer. For detection of T cell subsets, flow cytometry was performed. Briefly, 5×10^5 cells were stained for surface markers CD3, CD4, and CD8 with PerCP anti-human CD3, FITC anti-human CD4, and PE anti-human CD8 conjugated antibodies, washed twice and were then evaluated by BD FACSCalibur (BD, USA). Data were analyzed by FlowJo software and the frequency of the CD4+ T cells (CD3+CD4+) and CD8+ T cells (CD3+CD8+) was determined. The gating strategy was based on the acquisition of the $CD3^+$ T cell in the lymphocyte population followed by selection of the $CD4^+$ and $CD8^+$ T cells in $CD3^+$ gate.

Statistical analysis

Data were analyzed by IBM SPSS Statistics version 21. Descriptive data were presented as mean and standard deviation. Comparison of serum Ig level and T cell subsets between two groups was conducted by Mann-Whitney U test. A p-value less than 0.05 was considered as statistically significant.

RESULTS

Twenty-eight male patients with HA were investigated and compared with 20 age-matched healthy males. Mean age of the patients and control group was 30.6 ± 8.5 and 29.5 ± 6.2 years, respectively. Overall, the in-

Table 1. Comparison of T cell subsets and serum immunoglobulin level in patients with hemophilia and healthy individuals.

Variable	Patients (n = 28)	Controls (n = 20)	p-value	Inhibitor (-) (n = 21)	Inhibitor (+) (n = 7)	p-value
Serum IgM (g/L)	1.12 ± 0.565	0.958 ± 0.358	0.215	0.98 ± 0.48	1.43 ± 0.73	0.105
Serum IgA (g/L)	2.54 ± 1.05	2.24 ± 0.826	0.293	2.52 ± 1.2	2.74 ± 0.61	0.352
Serum IgE (IU/mL)	257 ± 454	139 ± 172	0.274	261.26 ±521.78	277.3 ±247.28	0.254
Serum IgG (g/L)	14.35 ± 3.60	12.4 ± 1.72	0.014 *	14.43 ± 3.68	14.6 ± 3.09	0.832
Serum IgG_1 (mg/L)	8,106 ± 1,917	5,890 ± 1,101	< 0.001 *	8,119.67 ± 1,968.56	8,037 ± 1,614.27	0.894
Serum IgG_2 (mg/L)	5,096 ± 2,001	5,296 ± 1,139	0.559	5,068.9 ± 2,114.21	4,913.29 ±1813.51	0.959
Serum IgG_3 (mg/L)	801 ± 418	639 ± 200	0.076	751.82 ± 470.3	973.6 ± 251.53	0. 111
Serum IgG_4 (mg/L)	786 ± 487	774 ± 581	0.937	775.42 ± 525.78	776.94 ± 415.38	0.811
T cell frequency (%)						
$CD3^+CD4^+$	28 ± 14.8	35.4 ± 12.6	0.076	28.27 ± 15.91	27.25 ± 12.08	0.75
$CD3^+$ $CD8^+$	20.4 ± 11.6	22.9 ± 9.6	0.413	20.66 ± 12.64	19.44 ± 9.18	0.614
CD4/CD8 Ratio	1.50 ± 0.52	1.65 ± 0.56	0.346	1.56 ± 0.58	1.38 ± 0.33	0.474

*** Statistically significant.**

hibitor was present in seven patients (25%) among them, 4 patients have inhibitor level between 0.5 - 4.99 Bethesda units and 3 patients have inhibitor level > 5 Bethesda units.

Comparison of T cell frequency and serum immunoglobulin level in patients and controls is demonstrated in Table 1. Accordingly, among all serum immunoglobulin classes, the concentration of IgG antibody was significantly higher in patients compared to controls (14.35 ± 3.60 g/L, vs. 12.4 ± 1.72 g/L, p = 0.014). Of all IgG subtypes, the IgG_1 subclass was considerably higher in hemophilia patients than healthy individuals (8,106 ± 1,917 mg/L vs. 5,890 ± 1,101 mg/L, p < 0.001. The frequency of $CD4^+$, $CD8^+$ T cells, and CD4/CD8 ratio showed no statistically significant differences between patients and controls (p > 0.05).

There was no significant difference between patients with and without inhibitors regarding serum immunoglobulin level, different IgG subtypes, the frequency of CD4+ and CD8+ T cells as well as CD4/CD8 ratio (p > 0.05) (Table 1).

DISCUSSION

There is a reciprocal relationship between the immune system and coagulation cascade indicating that they are closely linked together and their balance optimizes response to injury and pathogen invasion. As a result, dysregulation of the coagulation system can affect the entire balance and may result in a wide range of immune deficiency related disease exposure [11].

Intravenous FVIII infusion on demand or prophylaxis is the current standard care for HA patients. The main life-threatening complication after exogenous FVIII administration is the production of anti-FVIII neutralizing antibody, also known as "inhibitor" which occurs in 20 - 30% of patients [12]. Several studies describe that exogenous FVIII can trigger an immune response which is a T cell-mediated immunity that depends on processing and presentation of a part of the FVIII antigens by antigen-presenting cells (APCs), such as macrophages, dendritic cells, and B cells in the context of major histocompatibility complex (MHC) molecules [10,13]. This issue emphasizes the importance of the adaptive immune system in the production of inhibitor, where the patients with inhibitors cannot use concentrate products and need expensive bypass methods [14]. In this study, we compared the cellular and humoral immune system in patients with hemophilia A and healthy controls as well as in hemophilia patients with and without inhibitor production. Our results showed that serum IgG, especially the IgG_1 subtype, was significantly higher in hemophilia patients than the control group. The frequency of $CD4^+$ as well as $CD8^+$ T cells did not significantly differ between patients and control group. There was no significant difference between patients with and without inhibitors regarding serum immunoglobulin level, different IgG subtypes, the frequency of CD4+ and CD8+ T cells as well as CD4/CD8 ratio.

In a study by Jardim et al., it was shown that previously untreated patients (PUPs) with HA have a distinct immunological profile before receiving exogenous FVIII characterized by the production of pro-inflammatory cytokines like IL-8, IL-6, IL4, I-10, IL-2, and IL17-A [15].

Consistent with our results, previous studies have demonstrated that during exposure to factor products, patients with HA still represent this pro-inflammatory immunological status, associated with production of IL-2, tumor necrosis factor-α (TNF-α), interferon-γ (IFNG-γ), and anti-FVIII IgG_1 antibody with no inhibitory activity [15,16]. Interestingly, patients with HA who were positive for inhibitor (HAα-FVIII(+)) present an anti-inflammatory/regulatory immunological profile accompanied with dominant anti-FVIII IgG_4 antibody produc-

tion [15-17]. According to research by Irigoyen MB on hemophilia A patients, IgG_4 was predominant in low and high inhibitor level. The study showed 40% of patients without inhibitor and 93.7% of patients with inhibitor had antibody against factor VIII [18]. In a prospective study by Miller et al. in 17 hemophilia centers, inhibitors were shown in hemophilia A patients including only IgG_1 and IgG_4 subclasses [19]. In another cohort study by Whelan et al., IgG_4 and IgG_1 subclasses were the most abundant IgG subclasses in patients with FVIII inhibitors and IgG_4 was completely absent in patients without FVIII inhibitors and in healthy subjects [20].

Based on the above-mentioned studies, our data are compatible with significant higher IgG_1 level in hemophilia patients. However, IgG_4 level was not different between patients with and without inhibitors. This discrepancy might be due to the difference in an ethnic group, genetic background as well as a limited number of our patients with inhibitor.

CONCLUSION

Patients with hemophilia A have high levels of serum immunoglobulin especially IgG_1 with no change in the frequency of $CD4^+$ and $CD8^+$ T cells. Therefore, further larger study along with close observation and evaluation of the presence of serum inhibitor is recommended every 3 months.

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Declaration of Interest:
The authors declare that they have no conflict of interest.

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