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The Effects of Recombinant Human Erythropoietin (rHuEPO) on Antihuman Lymphocyte Antibodies Titer in Sensitized Rats

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Abstract

Erythropoietin (EPO) was first known as a factor for red blood cell proliferation and differentiation. Recent studies show the effects of EPO on immune system. In this study the antihuman leukocyte antibody (anti-HLA) titer were determined in five groups of rats, which had been sensitized with human lymphocyte. Also, the effects of stimulation's frequency and dose of recombinant human erythropoietin (rHuEPO) on anti-HLA antibody titer were studied. Two groups of rats received 20 and 100 IU/kg rHuEPO respectively, after twice sensitization with human lymphocyte. The other two groups were given 20 and 100 IU/kg rHuEPO, but after three times sensitization with human lymphocyte. Control group did not receive rHuEPO. Microlymphocytotoxicity method was used to detect anti-HLA antibodies. The results show that the anti-HLA antibody titer has been decreased significantly compared to control group. This statistically significant decrease was seen in groups, which received 100 IU/kg rHuEPO, and also in those, which received 20 IU/kg after 2 antigenic stimulations. This could be due to the effects of rHuEPO on the number or the activity of B cells and T cells. Moreover, the dose of rHuEPO, length of treatment and the level of sensitization with human lymphocyte might affect anti-HLA antibody titer.

Keywords: Anti-HLA antibody titer; rHuEPO; Lymphocytes

Introduction

Erythropoietin (EPO) is a glycoprotein hormone produced in kidneys in response to tissue hypoxia and is the main regulator of red blood cell (RBC) production in the body as well [7]. It is now available by recombinant DNA technology [4]. Recombinant Human erythropoietin (rHuEPO) is used in renal diseases to treat anemia [4,10]. Recent data indicate that rHuEPO may also have considerable effects on immune system. Pfaeffl *et al.* found a significant decrease in CD3, CD4 and CD8 after 18 months administration of rHuEPO compared to control group values, although the CD4/CD8 ratio was increased [15]. Other studies showed that rHuEPO have some effects on both humoral and cellular immunity as an immuno-modulatory factor [3,12]. The increasing rate of rHuEPO administration in dialysis patients was shown

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changes in antibody titers [9,10]. In other experiments, it was shown that rHuEPO affects immune cells [2] and cytokine production [6]. Dose of administration [12], length of treatment [12,17] and the rate of presensitization of the patients with renal deficiency [18] might influence the rHuEPO effects on immune system as well as antihuman leukocyte antibody (anti-HLA) titer.

The purpose of the present study was to examine the effect of rHuEPO on anti-HLA antibody titer as well as the use of different doses of rHuEPO. Furthermore, we wanted to find out if there exists any relationship between the effects of rHuEPO and the rate of sensitization with human lymphocyte.

Material and Methods

Animals

Sixty female, *Sprague Dawley*, rats of the inbred strain (Pasture Inst., Tehran, Iran) was allowed to acclimatize to the laboratory environment for one week. Then the experiment was started at seven weeks of age when the animals had a body weight of 240-290 g. A standard rodent feed in pellet form and tap water *ad libitum* were available throughout the study.

Animal Sensitization

All rats were immunized with HLA antigens, using 0.1 ml intrapritoneal injection of human lymphocyte suspension. Standard protocol was used for separating lymphocytes [5]. Lymphocytes were prepared from 15-20 ml of peripheral blood from a unique donor. The mean number of lymphocyte was adjusted to1270/µL.

Erythropoietin

rHuEPO (Eprex: Amgen Corp, Thousand Oaks, CA) was administered subcutaneously at two doses of 20 and 100 IU/kg body weight twice a week for six weeks. The control rats received vehicle solution instead of the administrated rHuEPO.

Rats grouping and Treatments

Rats were divided into five groups. One group was considered as control and immunized on days 0, 22, and 67. The first two groups (I and II) were immunized on days 0, 22 and anti-HLA antibody levels were measured 4 days after the last injection. Then the rats received 20 and 100 IU/kg rHuEPO for six weeks, respectively. Group III and IV were immunized on days 0, 22 and 67; the anti-HLA antibody titers were measured 3 days after last injections. Afterwards, they received the same doses of rHuEPO. Anti-HLA antibody levels were measured in all rats the day after the last injection of rHuEPO.

Serum Samples

Blood samples were taken from orbital sinus of rats. Serum samples were separated and stored at −20°C.

Antibody Determination

NIH (National Instituted of Health) microlympho-cytotoxicity method was used to detect anti-HLA antibodies [8]. Rats' sera were thawed using Hanks solution made serial dilutions. 1 µl of each dilution was dispensed into a known HLA plate well. For each group of sera a positive and a negative control serum were used. 1 µl of lymphocytes were added to each well and after 30 min incubation in room temperature, 5 µl of rabbit complement (Razi Inst., Karaj, Iran) was added and incubated one h. Finally 2 µl eosin dye (Merk, Germany) and 5 µl formalin (Merk, Germany) was added. Results were observed by using a phase contrast and inverted microscope. The reactions were scored using a rough scale to facilitate the estimation of cell killing based on the changes in viability between the negative control and test wells [8].

Data Analysis

Comparison of means, before and after the treatment with rHuEPO, was performed using one-way analysis of variance followed by Tukey test as a *post hoc* test. Statistical significance was taken as $p<0.05$.

Results

rHuEPO Effect on Antibody Titer after Twice Antigenic Stimulation

Mean antibody score in control and test groups (I and II) are shown in Figure 1. Mean antibody score differences in these groups were not significantly different before using rHuEPO ($p>0.05$). By using rHuEPO, the differences were significant ($p<0.05$). The differences also were significant between test groups.

rHuEPO Effect on Antibody Titer after Three Antigenic Stimulation

Mean antibody score in control and test groups (III and IV) are shown in Figure 2. Mean antibody score

differences in these groups were not significantly different before using rHuEPo ($p>0.05$). After using rHuEPO, the differences were significant between the control group and group IV (100 IU/kg; $p<0.05$) but not in group III (20 IU/kg; $p>0.05$). There was also significant differences between group III and IV statistically ($p<0.05$).

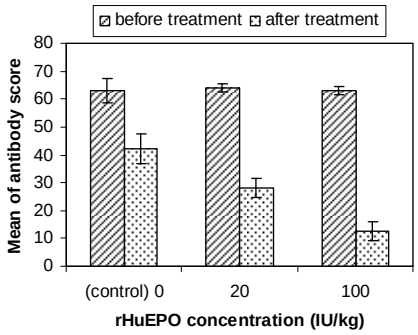

Figure 1. Comparison between mean antibody score in control and test groups before and after treatment with rHuEPO after twice stimulation with human lymphocyte. Lines on the charts represent means±SEM.

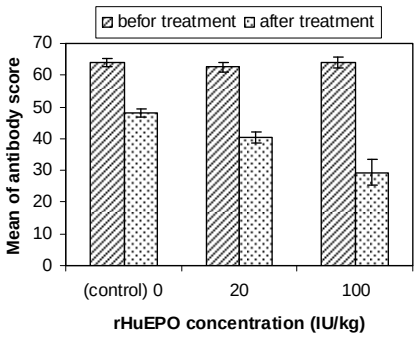

Figure 2. Comparison between mean antibody score in control and test groups before and after treatment with rHuEPO after three times stimulation with human lymphocyte. Lines on the charts represent means±SEM.

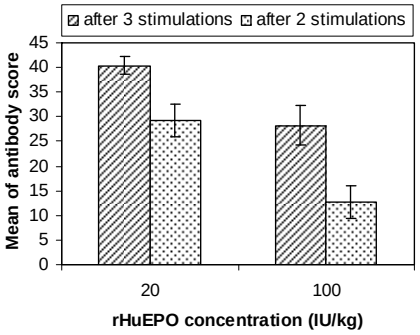

Figure 3. Means antibody score comparison in test groups with different frequencies of stimulation with human lymphocyte. Lines on the charts represent means±SEM.

rHuEPO Effect on Antibody Titer and Its Relation to the Number of Antigenic Stimulation

Two groups of rats were immunized twice and the other two groups were immunized three times with human lymphocyte. Six weeks after rHuEPO injection the second group (with 100 IU/kg and twice immunization) showed the most statistical significant reduction of antibody titer ($p<0.05$) (Fig. 3).

Discussion

EPO reduces multiple blood transfusion needs in patients with renal failure. It not only recovers anemia but also decreases the production of anti-HLA antibodies resulting from blood transfusions [1]. It is not clear whether the anti-HLA antibody reduction in these patients is due to the effect of EPO on the immune system or the lower numbers of blood transfusions they receive. Complementary studies in healthy sensitized animals receiving fix amounts of white blood cells and different doses of rHuEPO clarify the reality and may help to find out the exact mechanism. In this study, rHuEPO administration for six weeks decreased anti-HLA antibody significantly compared to control group. This statistically significant decrease was seen in groups, which received 100 IU/kg rHuEPO, and also in those, which received 20 IU/kg after 2 antigenic stimulations.

In recent studies, reduction in anti-HLA antibody titer was found in dialysis patients who receive EPO [1,10,11]. The exact mechanism is unclear. The observed results could be due to EPO effects on decreasing lymphocyte subpopulations or decreasing B cell and T cell functions [16]. Barany *et al.* proposed that the involved mechanism might be due to the direct effects of rHuEPO on immune system [3]. Imiela *et al.* found out decreased B cell differentiation and T cell antigenic response as a reason for this observation [12]. Furthermore, it was reported that absolute number of B cells in circulation was reduced during the initial period of rHuEPO therapy, with a return to baseline levels after 18 weeks of treatment [15].

The observed reduction might be result of rHuEPO dose. In this study 100 IU/kg rHuEPO was more effective than 20 IU/kg in decreasing antibody titer. It was thought that tissue uptake clearance of erythropoietin changes as a function of its dose [13]. The larger doses or more injection may upregulate EPO receptors and increase tissue absorptions [13]. Therefore, the 100 IU/kg rHuEPO may be more effective than 20 IU/kg on target tissues. Our data are in accordance with those researchers who believe that immunosuppressive activity of rHuEPO can be observed with the doses achievable in the serum during

therapy [12]. In Vitro studies on rHuEPO indicate that the hormone may stimulate Ig production by B cells [14]. However, the effects were seen with concentration much higher than those used in our study and than pharmacological concentration. These findings also show that the pharmacologic response to rHuEPO is a function of dose and dosing regime. So, it seems necessary to establish the best dose to make sure about the immunomodulatory effects of EPO.

On the other hand, the observed results could be due to the length of treatment with rHuEPO. It has been reported that rHuEPO caused a reduction on T and B cells response in first 3-6 weeks of rHuEPO administration and then an increase of response was observed [12]. Therefore, our results might be due to the short-term period of treatment with rHuEPO.

It should be mentioned that the frequency of antigenic stimulations could increase presensitization. There are some more probable reasons; such as the presence of lymphocyte subpopulations that contain not only B cells but also T helper cells. These cells increase antibody response even to minute amounts of external HLA antigens [11]. These cells accelerate the antibody response even in the presence of low doses of HLA antigens. Vella *et al.* reported that rHuEPO could not decrease anti-HLA titer in patients who had been highly presensitized by multiple pregnancy, blood transfusion or previous transplantation [18]. Regarding these data; it is possible that the lower reduction in antibody titers in group III is caused by highly activation of their immune system. In this situation rHuEPO was not effective on reduction of lymphocytotoxic antibody titer.

Immunomodulatory effects of EPO are a new perspective in the field of immunology. In this study a reduction of anti-HLA antibody titer was observed. However, the dosing regime, the length of treatment or the level of presensitization of rats may influence the results. More studies are needed to clarify the exact mechanism.

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