# Effects of Recombinant Human Erythropoietin Pretreatment on Anti-HLA Antibody Titer: A Preclinical Experience in Rats

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## ABSTRACT

**Background:** Erythropoietin (EPO) was first known as a factor for red blood cell proliferation and differentiation. New studies show the effects of EPO on immune system. **Objective:** In this study, the effects of pretreatment with recombinant human erythropoietin (rHuEPO) on the anti-human leukocyte antibody (anti-HLA) titer were determined. **Methods:** Three groups of rats were sensitized with human lymphocytes. Two of the groups were given 20 or 100 IU/Kg rHuEPO after two sensitizations with human lymphocytes. Control group did not receive rHuEPO. Microlymphocytotoxicity method was used to detect anti-HLA antibodies. **Results:** Treatment with rHuEPO caused a significant decline in anti-HLA antibody titer compared to control group. Also, pretreatment with rHuEPO suppressed antibody response after repeated antigenic stimulation. **Conclusion:** Such results could be due to the effects of rHuEPO on the number or the activity of the B and the T cells. Moreover, the dose of rHuEPO and the length of treatment might affect anti-HLA antibody titer.

**Keywords: Anti-HLA Antibody Titer, rHuEPO, Lymphocytes**

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## INTRODUCTION

Erythropoietin (EPO), a 34-Kd-glycoprotein hormone is the main regulator of red blood cell production (1). Recombinant human erythropoietin (rHuEPO) was introduced in 1989 to treat the anemia associated with chronic renal failure (2). Recent data indicate that aside from its well-known stimulating effects on red cell production, EPO may also have immunomodulatory properties (3). Nevertheless, studies addressing the influence of rHuEPO on the immune system are still limited and their findings inconsistent. The increasing rate of rHuEPO administration in dialysis patients showed decreases in anti-HLA antibody titers (4). While, *in vitro* production of some antibodies increased upon administration of rHuEPO (5). Studies in patients have shown that rHuEPO therapy may decrease the absolute number of circulating T cells (6). It seems that the dose (3) or the length of treatment (7) might influence its effects on immune system and on antihuman leukocyte antibody (anti-HLA) titer.
Since the immune system of rat is somewhat similar to that of humans, it is appropriate to use it for studying the effects of rHuEPO on the immune system. The aim of our current study was to assess the effects of rHuEPO on anti-HLA titer following treatment of sensitized rats for 6 weeks with two doses of rHuEPO, as compared to controls. Another objective was to evaluate whether or not the rHuEPO therapy could suppress antibody response to repeated antigenic stimulation.

## MATERIALS AND METHODS

**Animals.** 36 female *Sprague Dawley* rats of the inbred strain (Pasture Inst., Tehran, Iran) were allowed to acclimatize to the laboratory environment for one week. Then the experiment started at seven weeks of age when the animals had a body weight of 240-290g. A standard rat chow and tap water were available *ad libitum* throughout the study.
**Animal Sensitization.** All rats were immunized with HLA antigens, using 0.1 ml intraperitoneal injection of human lymphocyte suspension. A standard protocol was used for separating lymphocytes (8). Lymphocytes were prepared from 15-20 ml of peripheral blood from a unique donor and their number was determined. The mean number was adjusted to 1270/µL.
**Erythropoietin Administration.** 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 rHuEPO.
**Treatments.** Rats were divided into three groups. One of the groups was considered as a control group. All rats were immunized on days 0 and 22 of the study. HLA antibody levels were measured 4 days after the last injection. Then two groups of rats received 20 and 100 IU/Kg rHuEPO for six weeks, respectively. Anti-HLA antibody levels were measured in all of the rats one day after the last injection of rHuEPO. Afterwards all of the rats were immunized on 67th day for the third time and anti-HLA antibody titers were measured 3 days after the third injection of the antigen. Blood samples were taken from the orbital sinus of the rats. Serum samples were separated and stored at -20°C.
**Antibody Determination.** NIH (National Institutes of Health) microlymphocytotox-

icity method was used to detect anti-HLA antibodies (9). Rats' sera were thawed and serial dilutions were made using Hanks solution. 1µl of each dilution was dispensed into a known HLA plate well. For each group of sera a positive and a negative control serum were also used.Then 1 µl of lymphocytes was added to each of the wells and after 30 minutes incubation at room temperature, 5 µl of rabbit complement (Razi Inst., Karaj, Iran) was added to each of the wells and incubated for one hour. Finally 2 µl of eosin dye (Merk, Germany) and 5 µl of formalin (Merk, Germany) were 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 change in viability between the negative control and the test wells (9).

**Statistical 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

Mean antibody scores for the duration of the study in the control and the test groups are shown in Fig. 1. Mean antibody score differences in these groups were not significantly different before using rHuEPO ($p>0.05$). A significant decline ($P<0.05$) in mean antibody score as compared with control groups was noted only after six weeks of rHuEPO therapy. The differences were also significant between test groups ($P<0.05$). The mean antibody score was increased after the third antigenic stimulation. This elevation was less in groups that received 100 IU/Kg. However, the mean antibody score differences were significant between the test and the control groups ($P<0.05$). In this case, the mean antibody score differences were also significant between the test groups ($P<0.05$).

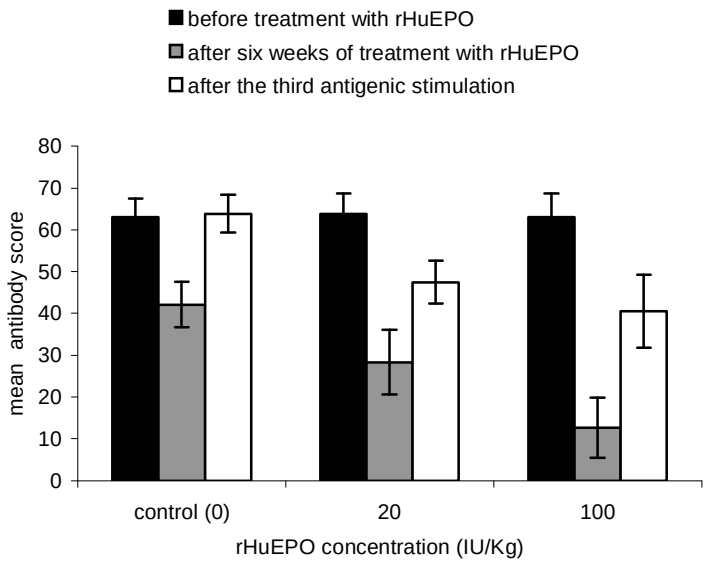


**Figure 1.** Comparison of mean antibody scores in the control and the test groups for the duration of the study. Vertical bars indicate SEM.

## DISCUSSION

Early reports suggested that rHuEPO therapy, through reduction of transfusion requirements and thus reduction of exposure to foreign antigens, might reduce the production of anti-HLA antibodies in renal failure patients (10). However, 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 reduced number of blood transfusions they receive. Complementary studies in healthy sensitized animals receiving constant amounts of white blood cells and different doses of rHuEPO clarify the situation and may help in finding out the exact mechanism. In this study, we found that treatment of sensitized rats with rHuEPO for six weeks correlated with a decrease in anti-HLA antibodies. We also demonstrated that administration of rHuEPO for six weeks could suppress the antibody response to antigen. This statistically significant response was more in the group which received 100 IU/Kg rHuEPO.

In some recent studies, reduction in anti-HLA antibody titer was found in dialysis patients who received rHuEPO (4, 11). The exact mechanism is unclear. Some workers propose that the mechanism might involve the direct effects of rHuEPO on immune system (12). The observed effects could be due to EPO effects on decreasing lymphocyte subpopulations or decreasing B and T cell functions (13). Imiela *et al.* found a decreased B cell differentiation and a decreased T cell antigenic response as a mechanism for this observation (3). It is possible that EPO treatment may influence lymphocytes because erythropoietin receptors have also been detected on lymphocyte- derived cell lines (14). In another investigation, it was shown that rHuEPO increases $CD8^+$ T cell apoptosis in hemodialysis patients (15).

The observed reduction might be a function of rHuEPO dose. In this study 100 IU/Kg rHuEPO was more effective than 20 IU/Kg. It was thought that tissue uptake and clearance of erythropoietin changes as a function of its dose (16).A larger doses or the number of injections may upregulate EPO receptors and increase EPO tissue absorption (16). Therefore, the 100 IU/Kg rHuEPO may be more effective on target tissues than 20 IU/Kg. Our data are in accordance with those of other investigators who believe that the immunosuppressive activity of rHuEPO could be observed with the doses achievable in the serum during therapy (3). In vitro studies on rHuEPO indicate that the hormone may stimulate Ig production by B cells (5). However, these effects were seen in concentrations much higher than those used in our study. These findings also show that the pharmacologic response to rHuEPO is a function of the dose. Therefore, it seems necessary to determine the appropriate dose to make sure about the immunomodulatory effects of EPO.

The observed results may also be due to the length of the treatment with rHuEPO. It has been reported that rHuEPO causes a reduction in T and B cells response in the first 3-6 weeks of rHuEPO administration and then an increase of response is observed (3). Therefore, our results might be due to the short-term period of treatment with rHuEPO.

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 regimen and the length of treatment may influence the results. More studies are needed to clarify the exact mechanism.

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