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S. PRUTCHI-SAGIV, ET AL. [2005] MED HYPOTHESES RES 2: 587-596.

ERYTHROPOIETIN AS AN IMMUNOTHERAPEUTIC AGENT: NEW USES FOR AN OLD DRUG?

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REVIEW

ABSTRACT. BROADLY BEYOND its erythropoietic functions, the biological effects of erythropoietin (Epo) have been demonstrated in other tissues outside the erythropoietic compartment, such as the brain, heart, and uterus. In addition, Epo has also been shown to mediate a wealth of effects on the immune system. These effects have been confirmed both in the cellular and humoral compartments of the adaptive response and also in the innate immune response. The most prominent effects of Epo shown in the adaptive immune response include: normalization of the CD4:CD8 T cell ratio, improvement of T cell functionality, increase of antibody production in response to antigen, and change in the cytokine profile. With respect to the innate immune reactions, Epo reduces macrophage and granulocyte-related oxidative stress and inflammation. Taken together, Epo may be considered as a candidate therapeutic agent applicable in a variety of cases of immune dysfunction and inflammation. Here we review the information accumulated to date regarding the immunological properties of Epo, but the molecular mechanisms underlying these effects still remain to be resolved.

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1. INTRODUCTION

Incessant research over the last ten years has led to the discovering of novel erythropoietin (Epo) physiological functions, broadly beyond erythropoiesis per se. Basically, major progress in treating anemias was due to the well known role of Epo in regulating red blood cells growth, differentiation and viability [1,2], the cloning of the gene in 1985 [3] and the introduction of recombinant human Epo (rHuEpo) into clinical practice. All of these allowed to treat anemias of various etiologies, including anemia of chronic kidney disease and cancer-related anemias [4-9]. The successful treatment with rHuEpo is characterized by an increase of hemoglobin (Hb) and hematocrit levels, leading to the correction of anemia and reduction of the need for blood transfusion. In addition, and correlated to the increase in Hb, an improvement of quality of life (QoL) is often observed [10].

The detection of the target receptor for Epo (EpoR) in cells other than erythroid progenitors, such as polymorphonuclear, megakaryocytes, endothelial, myocardial and neural cells [11-15] suggests that Epo has other biological (and potentially therapeutic) functions in addition to the involvement in erythropoiesis. Some of these effects include improvement in congestive heart failure [16,17] and neuroprotection [18-21]. Currently, a growing body of evidence indicates that rHuEpo may have an important effect on the immune system, including both the cellular and the humoral type responses. Although an increasingly number of studies has been published on this matter over the last ten years, the underlying mechanism of action of rHuEpo on the immune system is as yet unknown.

This article is focused on the immunological properties of rHuEpo, and it intends to review the information accumulated so far, and to point to open questions regarding its mode of action.

2. EFFECTS OF rHuEpo ON THE IMMUNE SYSTEM

The majority of studies concerning the effects of rHuEpo on the immune system derive from research carried out on renal disease patients undergoing hemodialysis (HD). These patients are often characterized by a state of immune dysfunction, including high levels of serum pro-inflammatory cytokines [22,23], changes in the number and function of peripheral blood lymphocytes [24-27] and reduced levels of antibody production [28]. Beneficial effects of Epo therapy have been documented primarily in these HD patients, as manifested in a wide range of immunological parameters, in both the cell-mediated and humoral compartments. Similar results were also obtained in multiple myeloma (MM) [29], congestive heart failure [30] as well as inflammatory bowel disease [31] patients. Results are summarized in TABLE 1.

However, it has been noted that in some of the patients who did not show any improvement in the hematocrit value, there was also no change in the immunological indices [32,33]. Consequently, the question was raised as to whether correction of the anemia in itself might be responsible for the improvement in immune responses, or whether Epo exerts any direct effect on the immune system. In a recent one-year clinical trial, Roman and colleagues [34] attempted at addressing this question. The effects of rHuEpo on HD patients with normalized hemoglobin levels were compared to a group of partially corrected hemoglobin level. The results revealed that certain aspects of the immune system, such as delayed-type hypersensitivity, CD8+ T cell percentage and T cell anergy were improved by normalization of the hemoglobin level with rHuEpo, but not in the partially corrected ones. In contrast, both groups had increased antibody production in response to tetanus toxoid vaccination. Thus, it seems that the mere elevation of red blood cell levels may be responsible for only part of the rHuEpo immunopotentiation function, and other mechanisms may also be operating.

3. ADAPTIVE IMMUNITY

3.1. CELL-MEDIATED IMMUNITY

Certain phenotypic changes in the lymphocyte subpopulations of HD patients have been observed following long-term rHuEpo treatment.

The major effect of rHuEpo therapy seems to

TABLE 1. MJOR EFFECTS OF rHuEpo ON THE IMMUNE RESPONSE.

ADAPTIVE RESPONSE		REFERENCES CITED
CELL-MEDIATED	Increase of the CD4:CD8 T cell ratio.	[25,33,35]
	Improvement of *in vitro* T-cell mitogenic proliferation.	[32,33,41]
	Decrease of the percentage of CD8+CTLA4+ T cells.	[25,43]
	Increase in T cells expressing co-stimulatory molecule CD28+.	[44]
HUMORAL	Increased antibody secretion to T cell dependent antigens.	[46-48]
	Increased production of IgM and thymidine uptake by a human IgM-producing cell line.	[51]
	Increased spontaneous production of IgE in MNC cultures from atopic subjects.	[49]
	Increased production of IgE in normal B cells when pre-activated with IL-4.	[49]
CYTOKINES	Decreased production of pro-inflammatory cytokines TNFγ and IL-6.	[25,62,77]
	Increased production of anti-inflammatory IL10.	[62]
	Increased production of Th1 cytokines IL2 and IL12.	[25]
	Increase in the *in vitro* production of IL-2.	[61]
INNATE RESPONSE		
POLYMORPHONUCLEAR CELLS	Reduced PMN cell number.	[71]
	Extent of PMN oxidative stress and inflammation.	[70-72]
	Improved PMN phagocytosis.	[38, 73,74]
MONONUCLEAR CELLS	Reduction in the serum levels of neopterin produced by activated macrophages.	[75]
	Increase in NK cells number.	[69]
	Decrease in NK cells number.	[38,35]
COMPLEMENT		
	Increase of complement receptor type 1 (CR1) on the surface of erythrocytes.	[53]

manifest in an increase of the CD4:CD8 ratio in the peripheral blood [25,33,35]. This effect is attributed mainly to a significant decline in the percentage of CD8+ T cells. Our own studies on rHuEpo-treated MM patients are in support of these observations [36].

On the other hand, the case of CD4+ T cells seems rather controversial. Several studies noted a slight increase in the percentage of CD4+ T helper cells [25], while others observed no change in this subpopulation [37]. In contrast to these observations, a total change [38,39] in both subpopulations numbers without significant change in the ratio was also reported.

Epo seems to have an effect not only on T cell numbers, but also on their functional capacity. In HD patients and in certain malignancies T cells show dysfunctions such as decreased proliferation after stimulation with mitogens [32] and a basal state of hyperactivity [26,40]. Following rHuEpo treatment, T-lymphocyte response to mitogen stimulation increased up to the levels observed in

healthy control subjects [32,33,41].

T cell dysfunction and even the anergy observed may be attributed to an increased expression of the inhibitory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4) observed in CD4+ and CD8+ in hemodialysis [25], as well as MM patients [42]. The molecule CTLA-4 cross-links the CD80/86 receptors in antigen presenting cells; causing down-modulation of T-cell responses. This molecule binds its receptors with over 20-fold higher avidity than the CD28 co-stimulatory molecule. In patients receiving rHuEpo, the percentages of CD8+ lymphocytes expressing CTLA-4 declined during one-year treatment [25,43], whereas T cells (mainly CD8+) expressing the co-stimulatory molecule CD28+ increased [44].

Recent findings by Trzonkowski and colleagues [44] may explain the decrease of peripheral blood CD8+ T cells after rHuEpo treatment. The study attributes the decrease of these lymphocytes to an increase in the intensity of apoptosis. The increased apoptotic activity seemed to be related to changes in the surface expression of tumor necrosis factor receptor I (TNFRI). According to this study, T cells showed high levels of chronic activation markers, such as Fas and TNFRI compared to healthy subjects, augmenting the chronic inflammatory state in HD patients. This state may be connected with the disturbed capacity of CD8+ T lymphocytes to undergo apoptosis. Indeed, rHuEpo treatment resulted in a gradually increasing ability of the CD8+ T cells to undergo apoptosis, along with the gradual decrease of TNFRI level.

An immunomodulatory role for Epo has also been suggested in MM. Clinical observations have shown that patients with advanced MM, treated with Epo for their anemia, lived longer than expected as based only on repair of the anemia [45]. Along with the observations on patients, an immunomodulatory effect of Epo has been demonstrated in a MM murine model. A study performed on MOPC-315, a murine myeloma model, provides evidence that rHuEpo treatment promotes the development of an effective anti-tumor-specific immune response, mediated via activated CD8+ T cells. Data from these studies on the murine experimental model suggested that anti-myeloma immunological reactivity of Epo is involved in augmentation of tumor regression in 50% of Epo-treated mice, prevention of bone disease in 20% and significant prolongation of survival of the tumor-bearing mice [29].

3.2. HUMORAL IMMUNE RESPONSES

Epo therapy leads to improved humoral immune response, either directly, or via T-cell help. Supporting the first hypothesis, Birmingham and colleagues [46] measured antibody responses of HD patients after immunization to tetanus toxoid (TT), a T cell dependent antigen and pneumococcal capsular polysaccharide antigen (PA), a T cell independent antigen. rHuEpo therapy increased the response to TT but not PA; suggesting that rHuEpo enhances the response to T cell dependent antigens. In the same way, studies on predialysis and HD patients receiving rHuEpo treatment showed a significant increase in specific antibody responses to hepatitis B vaccine, (a T cell dependent antigen), in comparison to those who did not receive rHuEpo [47,48]. In support of this data, *in vitro* experiments demonstrated that rHuEpo enhances spontaneous IgE production by mononuclear cells (MNC) and purified B cells from atopic patients, yet only in the presence of T cells and monocytes. rHuEpo also induces IgE production in normal MNC and B cells when activated by IL4 [49]. These facts seem to indicate that Epo enhances antibody secretion probably by inducing T helper cells to secrete Th2 cytokines and thereby augmenting the response of B cells.

In contrast, there is also evidence pointing to direct effects of rHuEpo upon B cells. Schaefer and colleagues [50] demonstrated enhanced basal and mitogen-stimulated immunoglobulin production by cultured peripheral MNC of dialysis patients after Epo treatment. Furthermore, when MNC were directly incubated with Epo, production of IgG, IgA and IgM was enhanced. The stimulation of B cells in these cases was not mediated by either T helper cells or monocytes, suggesting a direct interaction between B cells and

rHuEpo. In addition, experiments performed in serum-free medium, in order to rule out the effects of other cytokines and hormones that are present in the serum, show that rHuEpo directly stimulates B cells and enhances their proliferation and the production of Ig. Noteworthy, this study showed that Epo stimulated activated B cells, but not resting B cells. In the same line, high doses of rHuEpo enhanced *in vitro* Ig production and proliferation of various plasma cell lines, as well as human plasma cells generated *in vitro* [51,52].

Furthermore, rHuEpo therapy increases the expression of complement receptor type 1 (CR1) on the surface of erythrocytes [53]. This could result in an increased capacity to process circulating immune complexes that can serve as immunological signals. Processed immune complexes have their C3b sites degraded to the B cells CR2/CD19 complex. Depending upon the circumstances of ligation, this can either stimulate, or suppress B cell function. Increased erythrocyte CR1 levels by Epo therapy could thus enhance this process [54].

The augmentation of immunoglobulins in course of Epo therapy may be explained by the increase of helper CD4+ T cells and a decrease of suppressive CD152+ cells. A blockade of the reaction between the CD152 and the CD80/CD86 molecules by the CTLA4 Ig monoclonal antibody promotes synthesis of the cytokines known to be involved in the humoral response and switching the immunoglobulin classes [55]. Improved function of CD4+ cells was also indicated from an increased level of Th1 cytokines following Epo therapy. This effect may be related to suppressive CD8+CD152+ T lymphocytes. Increased secretion of IL12 by APC was partially mediated by reduced cross-linking of CD80/86 receptors on APC by CD152 ligand on CD8+ cells. This lower inhibitory signal also permitted sufficient presentation of antigens by APCs to CD4+ T cells and finally caused an enhanced secretion of IL2 that is produced by CD4+ cells. Epo did not alter late markers of inflammation such as CRP, C3 and C4. Therefore, the results indicate that the changes are not effects of inflammation, but are essential consequences of the influence of Epo on the lymphocyte subpopulations [25].

3.3. CYTOKINES

The involvement of pro-inflammatory cytokines in anemia is of extreme importance. It is maintained that the pathogenesis of chronic anemia is probably mediated via pro-inflammatory cytokines such as Interleukin 1 (IL-1), TNFα and interferon γ (IFNγ), secreted by activated T cells, monocytes and macrophages in acute and chronic inflammatory states [56]. In fact, *in vitro* studies indicate that these pro-inflammatory cytokines cause potent suppression of erythroid colony growth. IFNγ, particularly, promotes apoptosis of erythroid progenitor cells, consequently antagonizing the anti-apoptotic action of Epo [23,57,58]. In addition, pro-inflammatory cytokines such as IL-1 and TNFα may also inhibit the synthesis of endogenous Epo [59].

Epo therapy is believed to restore the imbalance of cytokines by decreasing the production of TNFα and IL-6, resulting in values characteristic of the healthy state. Concomitantly, there is an increase in the level of anti-inflammatory IL10, as well as the Th1 cytokines IL12 and IL2; the latter is a main cytokine for T-cell immunity that is decreased in T-cell cultures of uremic and HD patients [27,60,61]. Thus, the immunological conditions shifted from stimulatory to suppression processes [62].

The anti-inflammatory properties of Epo are also observed in the brain. Hence, studies on a mouse model of blunt trauma demonstrated that rHuEpo crosses the brain-barrier and markedly decreases the inflammatory infiltrate [63]. Similarly, Epo reduced the severity of manifestations in the rat experimental autoimmune encephalomyelitis (EAE) model, probably mediated by inhibition of the pro-inflammatory cytokines IL-6 and TNF-α [64] and of MHC II upregulation that occurrs throughout the disease [65].

Epo has also been shown to prevent inflammatory cell demise through pathways that involve phosphatidylserine (PtdSer) exposure, microglial activation, protein kinase B (Akt) [66] and the

regulation of caspases [67,68].

Interestingly, direct effect of rHuEpo on the production of IL-2 was indicated from *in vitro* studies, when physiological doses of rHuEpo (0.05 IU/mL) were added to whole blood cell cultures of the HD patients. This effect was only seen in cases of patients who produced low IL-2 levels in response to PHA, as opposed to patients who showed *a priori* high levels [61].

The elevated production of IL-2 during rHuEpo treatment may explain the observation that rHuEpo increased T cell response to mitogens and also improved function of cytotoxic T and NK cells [33,35]. The mechanism by which rHuEpo increases the level of IL-2 production by T cells *in vitro* is not understood. So far, there is no indication of EpoR existence on T cells. It has been proposed that the effect is due to stimulation of the common receptor subunit, the beta chain of IL-2R and EpoR. These receptors have conserved primary amino acid sequences in their cytoplasmatic domains; they show a striking degree of homology in the critical domain essential for signal transduction through the transcription factor STAT5 and they induce the cytokine inducible SH2 containing protein (CIS) [61].

Alongside with the above evidence pointing to immunopotentiation effects of rHuEpo, the fact that a few patients respond poorly to the drug needs to be taken in consideration. Several factors have been recognized to cause poor responsiveness to rHuEpo, such as iron deficiency and controversially, infection and inflammation conditions. All HD patients studied so far have shown a certain level of immune system over-activation and higher levels of inflammatory markers and pro-inflammatory cytokines. However, "poor" responders to rHuEpo treatment show significantly reduced CD28 expression on their T cells, enhanced IL10, TNFα and IFNγ secretion from MNC, as well as higher IL12 plasma levels, compared to the "good" responders group [23]. IL12 derived from monocytes activates T helper cells, leading to the production of IFNγ that may act as a pro-apoptotic factor of the erythroid progenitor cells. IL6 levels were increased in all patients with no difference between these two groups. Taken together, these findings suggest that the level of inflammation found in each patient may influence the efficacy of rHuEpo treatment, not only in increasing the level of hemoglobin, but also in enhancing functions of the immune system.

4. INNATE IMMUNITY

Studies on the immunological effects of rHuEpo therapy have addressed the innate immunity as well.

Results regarding the NK cell subpopulation vary in different studies. An increase in NK cells after treatment, compared to pretreatment level was observed by Collart [69], while others [35,38] reported a decrease in NK cells. Cytotoxic activity of NK cells did not change significantly [35]

Different observations were made on the effects of rHuEpo on PMN cells. Hence, several *in vivo* and *in vitro* studies demonstrated reduced PMN cell number, as well as the extent of PMN oxidative stress and inflammation [70-72]. The *in vitro* results indicate that Epo has a direct effect on PMN cells. In addition, these studies may explain the fact that *in vivo* effects of Epo on oxidative stress did not correlate with the improvement in Hb levels. Hence, primed PMN cells are known to contribute to an increased oxidative stress and inflammatory state as a result of the fact that they die of self-necrosis and at the same time recruit more PMN cells by cytokine release. A reduction in the priming state by Epo treatment increases their survival, decreases self-necrosis and reduces PMN recruitment to the circulation [70].

PMN cell phagocytosis also showed improvement after rHuEpo therapy [38,73] whereas the decreased chemotactic activity remained unchanged [74].

rHuEpo therapy also causes a progressive reduction in the serum levels of neopterin, a pteridine produced by IFNγ-stimulated macrophages, and commonly used as a marker of macrophage activity [75]. High levels of neopterin are found in HD patients and during the course of

many inflammatory diseases. Thus, therapy with rHuEpo reduces the macrophagic activation in these patients and enhances phagocytosis.

The effects on macrophages may also have an impact on wound healing, where they act as critical mediators by producing cytokines and growth factors essential to the healing cascade, and inducible nitric oxide synthetase (iNOS) in healing wounds. The ability of Epo to promote wound healing correlates with increased angiogenesis in granulation tissues, suggesting that the pro-healing function is associated with its stimulatory effect on proliferation and migration of endothelial cells during wound healing [76].

The finding that Epo receptors are expressed on PMN cells [13] and on macrophages [76], lends further support to the possible direct effect of Epo on these cells.

5. CONCLUDING REMARKS

It appears that Epo (either directly or indirectly) has measurable effects on the immune response. However, questions concerning the mechanism of action of Epo on the immune system still remain unclear. In addition, further evidence is required, to correlate the clinical efficacy of rHuEpo with its demonstrated immunomodulatory properties.

Since Epo receptor expression on T or B lymphocytes has not been reported, other cells expressing such receptors, in the immune system itself or in association with it might be involved in orchestrating the immunomodulatory effects of Epo (e.g., macrophages and monocytes).

Establishing the immunological effects of rHuEpo may open novel promising avenues in its clinical applications, in addition to treatment of anemia that is accompanying chronic diseases. Hence, it may be applicable in cases of immune dysfunction in other diseases manifesting immune abnormalities and in immunocompromised subjects.

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RECEIVED ON 7-21-2005.
ACCEPTED ON 8-17-2005.