

Erythropoietin Attenuates Lipopolysaccharide-Induced Splenic and Thymic Apoptosis in Rats

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Received April 4, 2005

Accepted July 5, 2005

On-line available August 5, 2005

Summary

Apoptosis of lymphoid tissues during sepsis is well documented and linked to the pathobiology of organ failure and death. In this study, we evaluated the effect of a single dose of recombinant erythropoietin (EPO) on thymic and splenic apoptosis in an endotoxic sepsis model. Young male Wistar rats were divided into 3 groups and administered intraperitoneally (IP) either normal saline; lipopolysaccharide (LPS) 10 mg/kg; or EPO (5000 U/kg) 30 min before lipopolysaccharide. Six hours following LPS administration animals were sacrificed. Apoptosis was assessed by hematoxylin-eosin staining, terminal deoxynucleotide transferase-mediated fluorescein-dUTP nick end labeling (TUNEL), and caspase-3 immunostaining. When compared with animals given LPS, animals pretreated with EPO displayed reduced splenic and thymic TUNEL positivity of 44 ± 3 ($p < 0.05$) and 143 ± 4 ($p < 0.05$) nuclei per high power field (hpf), respectively. Caspase-3 positivity was also significantly reduced in the spleen and thymus, with 31 ± 4 ($p < 0.05$) and 93 ± 3 ($p < 0.05$) positive stained nuclei per hpf, respectively. Serum nitrite levels were elevated in animals given lipopolysaccharide. Pretreatment with EPO attenuated the increase in nitrite levels; however, this did not reach statistical significance. We conclude that a single dose of recombinant erythropoietin can reduce thymic and splenic apoptosis associated with lipopolysaccharide administration.

Key words

Sepsis • Endotoxin • Lymphocyte • Erythropoietin

Introduction

Sepsis continues to be a leading cause of significant morbidity and mortality in intensive care units throughout the world. The pathophysiology of organ failure and death in patients with sepsis is still not very clear. Many patients with sepsis probably die due to an

inability to clear infection (Carcillo 2003, Hotchkiss and Karl 2003). This failure to clear infection may be associated with an immunosuppressive state, as septic patients shift from a state of immune overreaction to a state of anti-inflammatory immunosuppression (Hotchkiss and Karl 2003). Recent research suggests that apoptotic cell death is implicated in this

immunosuppression (Hotchkiss *et al.* 2003b). The loss of immune cells and production of anti-inflammatory cytokines or anergy may impair the ability of the host to control the infection (Hotchkiss and Karl 2003a, Hotchkiss *et al.* 2003b).

Apoptosis is an evolutionary conserved type of cell death, which has several features that distinguish it from necrosis. Apoptosis does not trigger inflammation, and the uptake of apoptotic cells by phagocytic cells leads to production of anti-inflammatory cytokines or anergy.

Animal and human studies have revealed apoptosis in various organs and tissues in sepsis (Wang *et al.* 1994, Ayala and Chaudry 1996, Hotchkiss *et al.* 1997). In humans dying of sepsis, extensive lymphocyte and gastrointestinal cell apoptosis was demonstrated (Hotchkiss *et al.* 1999a). Circulating lymphocytes of critically ill patients were shown to have signs of apoptosis (Schroeder *et al.* 2001).

Erythropoietin (EPO) is a glycoprotein that regulates red cell production. The primary action of EPO is to rescue erythroid cells from apoptosis, increasing their survival (Fisher 2003). It is widely used for the treatment of anemia in a variety of clinical situations. More recently, EPO has been shown to have neuroprotective (Ehrenreich *et al.* 2004) and other tissue and organ protective effects, mainly in ischemia-reperfusion (I/R) models (Carlini *et al.* 1999, Squadrito *et al.* 1999, Sharples *et al.* 2004).

Anti-apoptotic therapies for septic patients are not available at present. Agents and techniques utilized in most previous studies are not suitable for direct clinical use in septic shock. In this study, we hypothesized that the administration of recombinant EPO would protect the thymus and spleen from apoptosis induced by endotoxic shock. Serum nitrite levels were measured to evaluate the effect of EPO on nitric oxide production.

Methods

The experiments were performed in adherence to National Institutes of Health guidelines on the use of experimental animals. Young male Wistar rats, weighing 90-110 g, were housed in constant temperature at 14:10 h periods of light and dark exposure. After fasting overnight, the rats were randomly divided into three groups. The first group served as sham and received intraperitoneal saline (1 ml/kg), the second group, received intraperitoneal lipopolysaccharide (*Escherichia coli* LPS 055:B5 10 mg/kg, Sigma, St. Louis, USA,

n=12), in the third group, recombinant human erythropoietin alpha, (5000 U/kg, Roche) was given intraperitoneally 30 min before lipopolysaccharide administration. Six hours after administration of LPS, blood was taken by cardiac puncture for determination of nitrite levels and rats were sacrificed under ether anesthesia. The thymus and spleens were obtained through a midline laparotomy. Organs were cut to half and fixed in 10 % buffered formalin or immediately frozen at -80 °C.

Quantification of apoptosis

Tissues were fixed in 10 % buffered formalin overnight and embedded in paraffin after tissue processing. Serial sections (5 µm) were cut for histologic staining. Apoptotic cells were quantified using hematoxylin and eosin staining, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) and caspase 3 immunostaining. Apoptotic cells were quantified in five random x400 magnification fields. Quantification was performed in both organs by an investigator uninformed of the sample identity. On hematoxylin and eosin-stained sections, apoptotic cells were identified by the characteristic morphology of nuclear fragmentation (karyorrhexis) and cell shrinkage with condensed nuclei (pyknosis).

Caspase 3 immunostaining

Sections were mounted on poly-L-lysine-coated slides. The avidin-biotin-peroxidase method was performed using the primary monoclonal antibody against caspase 3 (1:100 dilution, Neomarkers, Fremont, USA). Briefly, the sections were deparaffinized and endogenous peroxidase activity was blocked using a 0.3 % solution of hydrogen peroxidase in PBS at room temperature for 10 min. After microwave treatment, primary antibody was applied for 30 min at room temperature and washed in PBS. Linking antibody and streptavidin-peroxidase complex (Neomarkers, Fremont, USA) were added consecutively for 10 min at room temperature and washed in PBS. The peroxidase activity was visualized with diaminobenzidine (Sigma, St. Louis, USA) applied for 5 min. Appropriate positive and negative controls were also labeled with the primary antibody.

In situ cell death detection

To detect DNA fragmentation in cell nuclei, TUNEL reaction was applied to the paraffin sections by

using a commercial kit (Roche, Cat.No. 1 684 817). After deparaffinization, the sections were treated with 20 mg/ml proteinase K for 10 min. After treatment with 0.3 % H₂O₂ in methanol for 10 min and 0.1 % Triton X-100 in 0.1 % sodium citrate, for 2 min on ice, the sections were incubated with TUNEL reaction mixture for 60 min at 37 °C. Further incubation with peroxidase conjugated antibody was performed for 30 min at 37 °C. The sections were stained with diaminobenzidine solution for 10 min at room temperature and then counterstained with hematoxylin.

Serum nitric oxide determination

To assay nitrite we used a modification of a previously published method (Baskin *et al.* 1997). Briefly, aliquots of 100 µl sample were mixed with equal

volumes of Griess Reagent mixture in a 96-well microtitre plate (Maxisorb Immunoplate, NUNC). After 10 min of incubation at room temperature, absorbance at 540 nm wavelength was measured in a microplate reader (Reader Model 230S, Organon Technica, Holland). A range of 2-fold dilutions of sodium nitrite (0-128 mM) in PBS was run in each assay to generate a standard curve.

Statistical analysis

Statistical analysis was done with the SPSS 11.0 software package. All values are expressed as mean ± SD. For comparison of apoptosis and nitrite levels between groups one-way ANOVA and post-hoc Tukey test were used. Differences were considered to be statistically significant if $p < 0.05$.

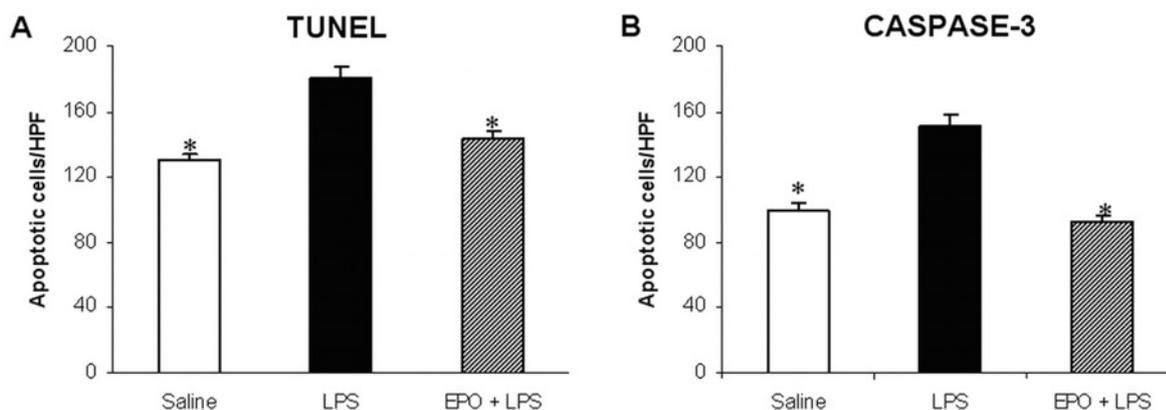


Fig. 1. EPO reduces thymic apoptosis. Thymi of rats given normal saline, LPS, or EPO and LPS were examined for apoptosis by TUNEL (A) and Caspase 3 immunostaining (B). * $p < 0.05$ when compared with LPS group. (EPO, erythropoietin, LPS, lipopolysaccharide, HPF, high power field)

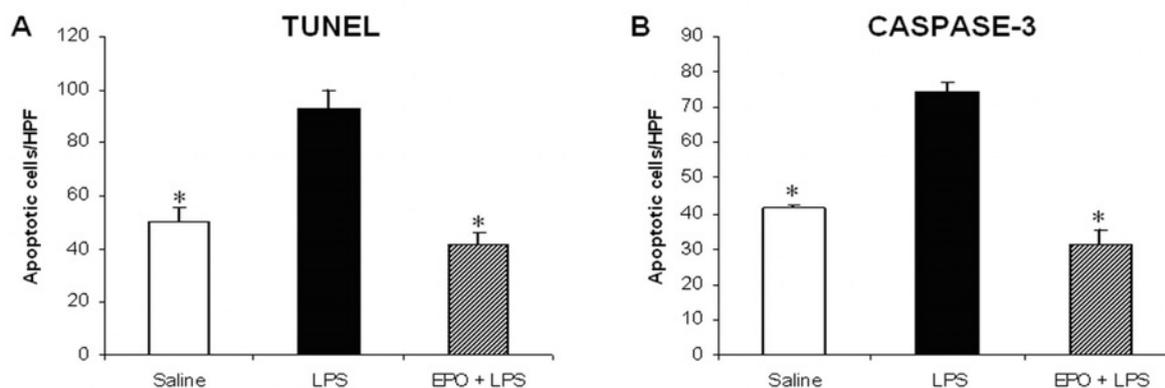


Fig. 2. EPO reduces splenic apoptosis. Spleens of rats given normal saline, LPS, or EPO and LPS were examined for apoptosis by TUNEL (A) and Caspase 3 immunostaining (B). * $p < 0.05$ when compared with LPS group. (EPO, erythropoietin, LPS, lipopolysaccharide, HPF, high power field)

Results

Three animals in the LPS group and one animal in the EPO + LPS group expired before end of the study at 6 hours. These animals were not used for the histopathologic studies. Conventional light microscopy of hematoxylin-eosin, TUNEL and caspase-3 immunostained specimens was performed by a pathologist who was uninformed to specimen identity. Five random fields were examined at x400 magnification, and cells demonstrating apoptotic changes or positive staining were counted. For assessment of apoptosis by hematoxylin-eosin staining, apoptotic cells were identified by the characteristic morphology of nuclear fragmentation (karyorrhexis) and cell shrinkage with condensed nuclei (pyknosis). Although this technique is not very sensitive, it is considered to be specific. By this technique, thymi and spleens from sham animals revealed an average of 120 ± 10 and 50 ± 5 apoptotic cells per high power field (hpf), respectively. Analysis of animals given lipopolysaccharide revealed an average of 184 ± 5 and 93 ± 7 apoptotic cells per field, respectively. In contrast, animals pre-treated with EPO displayed significantly reduced apoptosis with 136 ± 9 ($p < 0.05$) and 42 ± 4 ($p < 0.05$) cells demonstrating apoptotic changes, respectively.

TUNEL staining of spleen and thymus

Analysis of the LPS group animals ($n=9$) demonstrated an average of 108 ± 3 and 180 ± 7 TUNEL positive nuclei per hpf in the spleen and thymus, respectively. In contrast, those animals pretreated with EPO ($n=9$) displayed reduced average splenic and thymic TUNEL positivity of 44 ± 3 ($p < 0.05$) and 143 ± 4 ($p < 0.05$) positively stained nuclei per hpf, respectively (Figs 1, 2).

Caspase-3 staining of spleen and thymus

Analysis of LPS group animals ($n=9$) showed an average of 74 ± 3 and 151 ± 7 caspase-3 positive nuclei per hpf in the spleen and thymus, respectively. In contrast, animals pretreated with EPO ($n=9$) demonstrated reduced average splenic and thymic caspase-3 positivity of 31 ± 4 ($p < 0.05$) and 93 ± 3 ($p < 0.05$) caspase-3 positive nuclei per hpf, respectively (Figs 1, 2).

To assess intraobserver variation, the quantitation of apoptosis was performed twice by the same investigator who had no prior knowledge of the results achieved initially.

Effect of endotoxin administration and EPO administration on serum nitrite levels

Serum nitrite levels determined at 6 hours were low in the sham group of rats given normal saline. Nitrite levels were significantly elevated in the serum collected from lipopolysaccharide-treated rats when compared to the saline group (18.6 ± 7.5 vs. 46.4 ± 18.3 μM , $p < 0.05$). EPO reduced the increase in serum nitrite concentrations associated with LPS administration, but this did not reach statistical significance when compared with the LPS group (33.0 ± 15.0 vs. 46.4 ± 18.3 μM , $p = 0.15$) (Fig. 3).

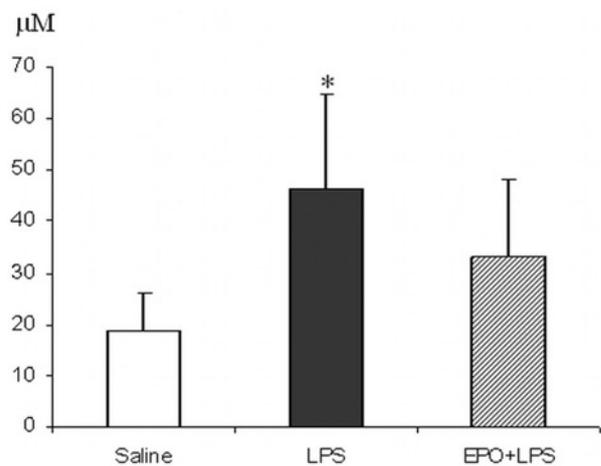


Fig. 3. Effect of EPO on serum nitrite levels in rats which were administered saline, LPS, or EPO + LPS. * $p < 0.05$ when compared with saline-treated group. (EPO, erythropoietin, LPS, lipopolysaccharide)

Discussion

The most important finding of the present study was the ability of a single dose of erythropoietin to protect against lipopolysaccharide induced thymic and splenic apoptosis.

Apoptosis of lymphoid tissues during sepsis is well documented and linked to the pathobiology of organ failure and death (Oberholzer *et al.* 2001, Ayala *et al.* 2003, Hotchkiss *et al.* 2003b). The thymus and spleen are seriously affected and lose a significant number of their lymphocytes during sepsis (Hotchkiss *et al.* 1999). The loss of these cells can lead to impaired cytokine production and antigen handling, resulting in an immunosuppressive state. Some hosts, unable to cope with the infection, will not survive as a consequence. Therefore, a clinical approach that aims to reduce apoptosis of lymphoid tissues appears to be warranted.

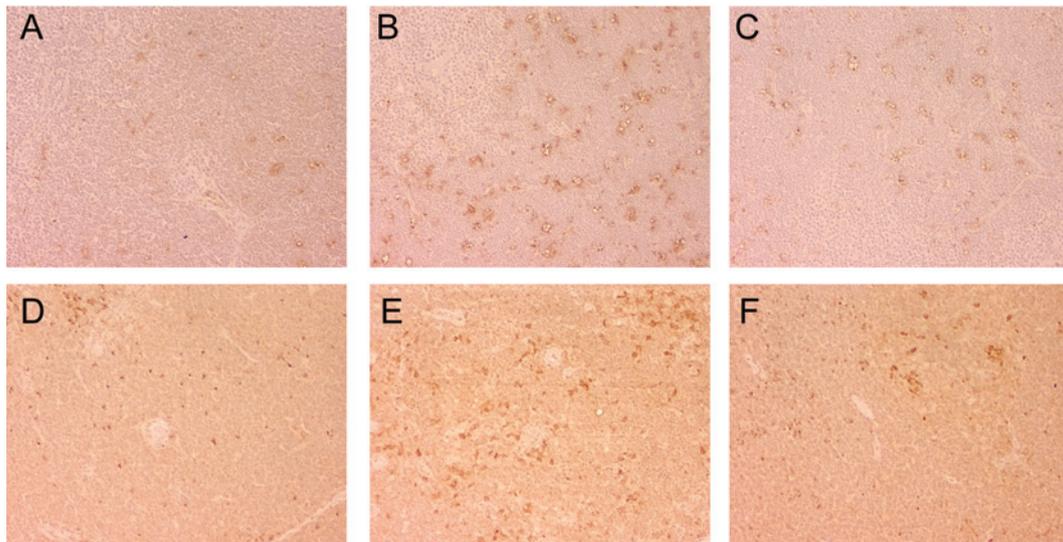


Fig. 4. A-C, Thymic tissue sections from sham and endotoxin shock rats were examined by the TUNEL method. A, Sham, B, LPS, and C, EPO + LPS group. D-F, Thymic tissue sections from sham and septic rats were stained by Caspase-3 immunostaining. D, Sham, E, LPS, and F, EPO + LPS. Note that a significant number of cells have positive staining in rats who received only LPS, whereas in animals which were pretreated with EPO, TUNEL and caspase-3 positive stained nuclei appear much less frequent. Magnification x100.

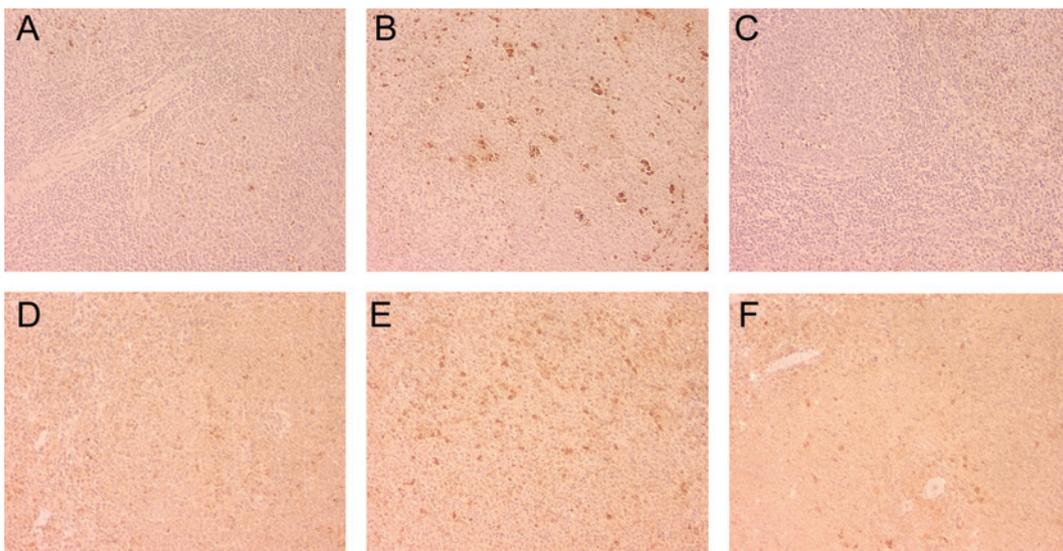


Fig. 5. A-C, Splenic tissue sections from sham and endotoxin shock rats were examined by the TUNEL method. A, Sham, B, LPS, and C, EPO + LPS group. D-F, Splenic tissue sections from sham and septic rats were stained by Caspase-3 immunostaining. D, Sham, E, LPS, and F, EPO + LPS. Note that a significant number of cells have positive staining in rats who received only LPS, whereas in animals which were pretreated with EPO, TUNEL and caspase-3 positive stained nuclei appear much less frequent. Magnification x100

Several experimental studies using the cecal ligation and puncture model of sepsis have established a survival benefit if apoptosis occurring during sepsis can be prevented or reduced. Transgenic mice in which Bcl-2 was overexpressed in T cells had protection against sepsis-induced T lymphocyte apoptosis in thymus and spleen, and improved survival (Hotchkiss *et al.* 1999b). The broad-spectrum caspase inhibitor z-VAD provided

similar protection (Hotchkiss *et al.* 1999c). Decreasing intestinal epithelial cell death *via* overexpression of Bcl-2 improves survival in septic mice (Coopersmith *et al.* 2002). Furthermore, overexpression of the serine/threonine kinase Akt, a key regulator of cell proliferation and death in lymphocytes, was found to prevent sepsis-induced apoptosis and improve survival (Bommhardt *et al.* 2004). Thus, targeting apoptosis in the

treatment of sepsis appears to be reasonable. The approach for reducing lymphocyte apoptosis in sepsis may include inhibition of caspase-3 activation, stimulation of anti-apoptotic protein expression like Bcl-2 or Bcl-XL, or inhibiting expression of pro-apoptotic proteins such as Bax or Bid (Oberholzer *et al.* 2001).

The prevention of apoptosis in animals receiving EPO in this study is in accordance with other studies that have established tissue protective and anti-apoptotic effects of erythropoietin. EPO protects against ischemia-reperfusion or hypoxic injury of the brain (Kumral *et al.* 2003, Prass *et al.* 2003), heart (Calvillo *et al.* 2003), and kidney (Sharples *et al.* 2004). It confers protection against development of multi-organ dysfunction (Abdelrahman *et al.* 2004), and was also shown to revert vascular dysfunction in a model of splanchnic artery occlusion shock (Squadrito *et al.* 1999).

The mechanisms by which EPO exerts its tissue protective effects are not entirely clear. Studies which established the neuroprotective role of EPO suggested that it may act at multiple levels, including limitation of the production of tissue-injuring molecules such as reactive oxygen species and glutamate, reversal of vasospasm, stimulation of angiogenesis, prevention of apoptosis, and modulation of inflammation. EPO may protect tissues by a combination of these mechanisms. The anti-apoptotic effect of EPO appears to play a major role in its tissue protective function. EPO may exert anti-apoptotic effects *via* regulation of the expression of genes involved in the apoptotic process. Studies have shown that EPO inhibits caspase 8-, caspase 1-, and caspase 3-like activities linked to cytochrome c release in models of neurological injury (Genc *et al.* 2004). In this study we found that animals given EPO had less immune staining for caspase 3. Pathways for apoptosis are modulated by the pro-apoptotic Bax family and anti-apoptotic Bcl-2 family of proteins. Bax protein was found to be increased in response to LPS administration and related to increased apoptosis (Munshi *et al.* 2002). EPO is known to decrease Bax mRNA expression and increase the expression of the anti-apoptotic gene Bcl-XL, a member of the Bcl-2 group of anti-apoptotic proteins (Genc *et al.* 2004). In this study we did not have the possibility to look at the expression of Bcl-XL or Bax, but it would be interesting to establish whether EPO use modulates their expression.

Involvement of nitric oxide (NO) in the early phase of hypotension and cardiovascular hyporeactivity

seen in septic shock has been demonstrated. Nitric oxide derived from vascular smooth muscle cells may contribute significantly to this hypotension and changes in cardiovascular reactivity (Liu *et al.* 1997). EPO may inhibit IL-1 β induced NO production by suppression of iNOS mRNA and its protein expression (Akimoto *et al.* 1999). We found elevated levels of nitrites in the serum of rats given lipopolysaccharide, similar to septic patients. EPO prevented the increase of nitrite levels, similar to the study of Squadrito *et al.* (1999), but in our study this effect did not reach a statistical significance.

Various aspects of EPO action on immunity have been investigated. The EPO receptor is expressed in lymphocytes (Heberlein *et al.* 1992) and treatment with EPO is reported to increase CD4 and CD8 cell counts without affecting the CD4/CD8 ratio. EPO also improves the impaired phagocyte activity in hemodialysis patients (Huraib *et al.* 1997). Plasma erythropoietin and IL-6 values are increased in critically ill children with sepsis or septic shock in comparison with controls (Krafte-Jacobs and Bock 1996). It is known that EPO production is increased by IL-6 presence, but the significance of this finding is not entirely clear at present.

We administered EPO before lipopolysaccharide administration. In order to determine if EPO can be clinically useful in the prevention of lymphocyte apoptosis in sepsis, its administration after a septic insult, such as endotoxin administration or cecal ligation and puncture, should produce similar effects. This remains to be investigated. Furthermore, it may be interesting to find out whether thymic and splenic nitric oxide synthesis is modulated by the administration of EPO during endotoxemia or other sepsis models.

In conclusion, we have demonstrated for the first time that erythropoietin protects against lipopolysaccharide-induced apoptosis in the rat thymus and spleen. Further research should examine the effects of EPO on apoptosis and survival in the sepsis model. Knowledge of whether EPO affects mainly B or T cells, and investigation of pro- and anti-apoptotic gene expression as mechanisms of EPO action are necessary.

Acknowledgements

We thank Dr. Kursad Genc for reviewing the manuscript. Supported by grant SBAG-AYD-439 (103S182) from the Scientific and Technical Research Council of Turkey (TUBITAK).

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Reprint requests

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