BenEFicial Effect of Erythropoietin Administration on Murine Infection with Trypanosoma congolense

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Abstract. The effect of erythropoietin treatment on Trypanosoma congolense infection in mice was studied. Survival rates of mice were dramatically improved by treatment with recombinant human erythropoietin (r-hu-EPO) when infected with 1,000 cells of T. congolense IL3000 (P < 0.05). All the untreated mice infected with T. congolense IL3000 died by day 9 of infection; however, 100%, 50%, and 25% of the mice treated with r-hu-EPO for 8 days survived to day 20, day 40, and day 60 of the parasitical infection, respectively. Anti-8-hydroxy-2'-deoxyguanosine antibody, a biomarker for oxidative damage of DNA, yielded positive reactions in the cytoplasm of the parasites recovered from the mice treated with r-hu-EPO. These results, taken together, indicate that erythropoietin administration is effective for the treatment of T. congolense infection.

INTRODUCTION

African trypanosome infection in humans and livestock results in sleeping sickness and Nagana, respectively. Although anemia is the major clinical and laboratory finding in these diseases, as well as malaria infection, little is known about the specific pathogenesis of the associated anemia. Anemia is mainly attributed to ineffective retention of ion and erythrocyte destruction by the mononuclear phagocytic system and ineffective erythropoiesis. Although the mechanisms underlying ineffective erythropoiesis during trypanosome infections are not known, it has been suggested that the anemia of trypanosomiasis is similar to the anemia of chronic disease (ACD) in humans. Patients with ACD have low concentrations of erythropoietin (EPO), the hormone responsible for stimulating erythrocyte production, relative to their degree of anemia. The administration of EPO to such patients resolves their anemia, showing that low concentrations of EPO contribute to the pathogenesis of ACD. It has therefore been suggested that the anemia of bovine trypanosomiasis is also associated with low plasma EPO and that EPO administration might resolve it, especially during the chronic stage when the response to trypanocide drug treatment is poor. In 1995, Buza and others reported that Trypanosoma congolense–infected calves exhibited increased in EPO concentrations in circulation, but nevertheless exhibited severe anemia. A similar observation in murine infection with virulent malaria (Plasmodium berghei) was published three decades ago. Since Buza and others have strongly suggested the presence of inhibitory factor/s for the erythroid response in trypanosome infection, no attempt has been reported of the administration of EPO for the treatment of anemia associated with protozoan infection. Hence, the potential use of an administration of high-dose exogenous EPO to resolve the virulent anemia caused by protozoan infection remains obscure. Thus, we examined the effect of EPO treatment on trypanosome infection in a mouse model. In addition, the effect of EPO was compared with pentamidine, a trypanocide drug.

MATERIALS AND METHODS

C57BL/6J female mice (CLEA Japan, Tokyo, Japan) at 7 weeks of age were infected with 1,000 cells of blood stream form T. congolense IL3000 (International Livestock Research Institute, Nairobi, Kenya) by intraperitoneal injection (day 0). The mice were treated daily with recombinant human erythropoietin (r-hu-EPO; EPOGIN S1500; Chugai Pharmaceuticals, Tokyo, Japan) at a dose of 5,000 U/kg body weight or pentamidine (pentamidine isethionate; Benembax 300; Chugai Pharmaceuticals, Tokyo, Japan) at a dose of 4 mg/kg body weight for 8 days from day 0 to day 7 of infection, and their survival rates, hematocrit values, and parasitemia were monitored. Hematocrit values and white blood cell (WBC) counts were determined using an autohematology analyzer (Cell tac α, MEK-6358; NIHON KOHDEN, Tokyo, Japan). Furthermore, to analyze the oxidative damage of parasite DNA, parasites were recovered from the circulation of the infected mice on day 7 after inoculation. Briefly, collected blood was mixed with 0.2 mol/L phosphate-buffered saline (PBS) containing 10% glucose (PSG) and centrifuged at 4,000 rpm for 5 minutes at 4°C. The washing was repeated three times. The pellet was resuspended with 0.1 mL of PSG and 0.1 mL of 3% fetal calf serum. The suspension was fixed with methanol for 10 minutes. Subsequently, 2 ng/mL of anti-8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker for oxidative damage of DNA, a monoclonal antibody (N45.1; MOG-20P; Japan Institute for The Control of Aging, Nikken SEIL Corporation, Shizuoka, Japan) was added and incubated for 45 minutes at 37°C. The sample was washed twice with PBS for 5 minutes. An anti-mouse IgG antibody (Alexa Flour 488 goat anti-mouse IgG, A11001; Molecular Probes, Eugene, OR) was added to the sample and incubated for 45 minutes at 37°C. The incubated sample was washed twice with PBS for 5 minutes. Parasite DNA was stained with propidium iodide (P1304MP; Molecular Probes) containing RNase A (10109142001; Roche Applied Science, Mannheim, Germany) for 10 minutes at 37°C. After washing with PBS, the sample was treated with 1% n-propyl gallate (102747; MP Biomedicals, Irvine, CA), an antioxidant, and observed with
a confocal laser microscope (DMRB/E, TCS NT; Leica Microsystems, Wetzlar, Hessen, Germany). To determine the expression pattern of EPO and EPO receptor mRNA in the kidney or liver of the infected animals, real-time quantitative polymerase chain reaction (PCR) was performed as follows. Total RNA was extracted from frozen kidney and liver of *T. congolense*-infected animals with or without EPO treatment by means of a TRI reagent Kit (Sigma, St. Louis, MO). The extracted total RNA was used for the real-time PCR analysis. Primers and the TaqMan probe for each gene and β-actin were designed using the primer design software, Primer Express version 1.5 (Applied Biosystems, Foster City, CA). The primer/probe sequences of the genes are summarized in Table 1. The quantification of all the gene transcripts was carried out by real-time quantitative reverse transcriptase (RT)-PCR with an ABI PRISM 7900 HT (Applied Biosystems). Templates for real-time PCR were obtained by reverse transcriptase reaction of total RNA. For RT-PCR reactions, the TaqMan One-Step RT-PCR master Mix Reagents Kit (Applied Biosystems) was used at 20 μL/tube as follows: the template (20 ng) was mixed with 2× Master Mix without uracil-DNA-N-glycosylase (UNG), 40× MultiScribe and RNase Inhibitor Mix, 200 nmol/L TaqMan Probe, and 900 nmol/L of each primer. Reaction conditions were one cycle at 48°C for 30 minutes and one cycle at 95°C for 10 minutes, followed by 45 cycles of the amplification step (95°C for 15 seconds and 60°C for 1 minute). The gene expression levels of EPO and the EPO receptor were calculated as gene expression rates, as previously reported. Briefly, the amount of each gene and β-actin mRNA in the samples was estimated with standard curves representing the log of the input amount (log starting cDNA molecules) as the x-axis and the threshold cycles as the y-axis. A relative standard curve (SC) for real-time PCR was used as a common set of samples that linked the experimental PCR plates together and permitted an overall analysis of the samples. Preparation and use of this SC as a quality control of the efficiency of amplification of the PCR plate is described elsewhere. The gene expression rate was obtained by normalizing the amount of each gene cDNA with that of β-actin.

The animals used in this study were housed in polycarbonate cages and maintained under a specific pathogen-free environment in light-controlled (lights-on, 0500–1900 hours) and air conditioned (temperature, 24 ± 1°C; humidity, 50 ± 10%) rooms. The mice had free access to standard laboratory chow (CA-1; CLEA Japan). Infectivity experiments were replicated at least three times to confirm the reproducibility of results. The Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine reviewed the protocols and confirmed that the animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine. Survival rates were analyzed statistically by the Gehan generalized Wilcoxon method. Other data were analyzed by Student t test.

**RESULTS**

As shown in Figure 1, survival of the *trypanosoma*-infected mice was dramatically improved by the treatment of erythropoiet (P < 0.05). All of the untreated mice infected with *T. congolense* IL3000 (1,000 cells) died by day 9 of infection, whereas all and 50% of the infected mice treated with 5,000 U/kg of r-hu-EPO for 8 days were still surviving 20 and 40 days after the parasitical infection, respectively. On day 60 after the infection, 25% of the EPO-treated mice still survived. In the control group, all of the mice infected with *T. congolense* died after increasing in parasitemia to a peak of approximately 2 × 10^8 cells/mL around day 7 after the inoculation (Figure 2A). With the increase in parasitemia, the control animals exhibited a decrease in hematocrit value by 30% and subsequently died (Figures 2A and B). No clinical side effects were detected in the mice that received r-hu-EPO without the parasite infection, except for a remarkable increase in hematocrit values (Figure 2B). Hematocrit values of the infected mice treated with r-hu-EPO and mice receiving r-hu-EPO without the infection similarly increased until day 6. However, there was a tendency for the hematocrit values in the infected mice treated with r-hu-EPO from day 9 to be lower than those in the mice that received r-hu-EPO. Furthermore, the hematocrit values of the infected mice treated with r-hu-EPO were much less than those of untreated control mice from day 16 onward. The increase in parasitemia in surviving animals occurred as several distinct peaks rather than as a sustained increase. In contrast, almost all infected mice without r-hu-EPO died at or just after the first peak of the parasitemia (Figure 2A). When two surviving females were mated with uninfected males on day 40 after the infection, one female delivered nine pups on day 73.

As shown in Figure 3, the effect of r-hu-EPO treatment on the *T. congolense* infection was equivalent or much more significant compared with pentamidine administration. However, no increase in hematocrit values in the infected animals was observed by pentamidine administration (Figure 4A). Although parasitemia similarly fluctuated the r-hu-EPO and pentamidine treatment in the infected mice, the parasitemia in the pentamidine group was significantly higher than in the r-hu-EPO group (Figure 4B; P < 0.05).

**TABLE 1**

Primer/probe sequence used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/probe</th>
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<tbody>
<tr>
<td>EPO</td>
<td>5'-CTACGTAGCCCTCACCTCAGTCTTT-3' (forward)</td>
</tr>
<tr>
<td>EPO receptor</td>
<td>5'-AGAGCCTTGCAAGAAATGTGCTGCTCCAGA-MGB-3' (reverse)</td>
</tr>
<tr>
<td>EPO</td>
<td>5'-FAM-CTCAGAAAGGATTGTGCTGCTCCAGA-MGB-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-CCCACGGCTTGGAAAGCTTG-3' (forward)</td>
</tr>
<tr>
<td>EPO</td>
<td>5'-CCTGGTCAGGCCTACATGACT-3' (reverse)</td>
</tr>
<tr>
<td>EPO receptor</td>
<td>5'-FAM-CAGGCTTCTCATACACGCTGCGGATGGA-MGB-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GCCACCAGATCCACAGAGT-3' (reverse)</td>
</tr>
<tr>
<td>EPO</td>
<td>5'-GCCACCAGATCCACAGAGT-3' (reverse)</td>
</tr>
<tr>
<td>EPO receptor</td>
<td>5'-FAM-ATCAAGATCATTGCTCTTC-MGB-3'</td>
</tr>
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As shown in Figure 5, 8-OHdG–positive reactions were located in the cytoplasm of the parasites recovered from the mice treated with r-hu-EPO, whereas few positive reactions were found in the parasites from the mice without r-hu-EPO treatment. The appearance of 8-OHdG in the EPO-treated group seems to induce enhancement of the oxygen tension in the circulation by EPO and a subsequent increasing of oxidative stress for the parasites.

Expression of EPO mRNA in the kidney increased transiently after the infection and decreased with infectious course (Figure 6A). On day 9 after the infection, EPO mRNA was not detectable in the kidney. In the infected mice receiving EPO treatment, transcription of EPO was not detectable during the treatment period but increased after the treatment on day 9. In the liver, the expression of EPO mRNA was down-regulated on day 3 and day 6 and recovered on day 9 to similar levels on day 0 (Figure 6C). Expression of the EPO receptor mRNA significantly declined with trypanosome infection with or without r-hu-EPO treatment. These results showed that the erythroid system of host animals was inhibited by T. congolense infection.

**DISCUSSION**

These results clearly show that the administration of r-hu-EPO to mice infected with T. congolense dramatically improves their survival rates (Figures 1 and 3). Although it is well known that one of the main features of the diseases caused by T. congolense infection is anemia, the majority of the infected mice did not exhibit anemia in this study. However, the mice that were killed exhibited a 30% reduction in hematocrit values on day 9; therefore, it seems that the rapid reduction in the hematocrit values of these animals, which did
occur in the acute phase of the trypanosome infection, between day 6 and day 9, was detected before their death in the examination performed every 3 days (Figure 2). It was not clarified whether administration of r-hu-EPO had a direct effect on the killing of the parasites in this study. Sustained elevation of the hematocrit values in the acute phase of trypanosome infection did seem to be effective in increasing survival in infected mice. It is plausible that an increase in free radical products as a result of a higher oxygen tension brought about by an elevated number of red blood cells in circulation might be harmful or even lethal to protozoan proliferation. In fact, the DNA of the parasites isolated from the EPO-treated animals did exhibit DNA damage caused by oxidative stress (Figure 5). In contrast, there was little DNA damage in the parasites from the EPO-untreated control mice. Furthermore, it is postulated that pretreatment of the parasites infection

**Figure 4.** Comparison of parasitemia (A) and hematocrit values (B) in *T. congolense*-infected mice with r-hu-EPO or pentamidine treatment.

**Figure 5.** Detection of oxidative damage of parasite DNA by immunostaining. **(A)** Parasites were recovered from the circulation of the infected mice on day 7 after inoculation and reacted with anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) monoclonal antibody, N45.1. **(B)** DNA of the parasites was stained with propidium iodide. **(C)** Differential interference contrast micrographs of the recovered parasites. **(D)** A merging of **A** and **B**. The parasites isolated from the EPO treated mice show a positive reaction against anti-8OHdG antibody, whereas little DNA damage is detectable in the parasites from the control mice (**A** and **D**).
with EPO would be effective for the prevention of the most pernicious disease symptom caused by trypanosoma. Because it was observed that the hematocrit values in the infected mice treated with r-hu-EPO were much lower than the r-hu-EPO treated and untreated control mice in the chronic phase, factors inhibitory to erythropoiesis seemed to be present in circulation in the infected animals. In support of this, the levels of EPO receptor transcripts in the bone marrow of \( T. congolense \)-infected N'Dama cattle (trypanotolerant) have been shown to be significantly higher than those in the bone marrow from infected Boran cattle (trypanosusceptible). In this study, smaller increases in the hematocrit values in mice infected with \( T. congolense \) subsequent to EPO treatment were observed compared with the untreated mice after EPO treatment. Thus, we examined the expression of EPO and EPO receptor mRNA in the kidney and liver in mice infected with \( T. congolense \) by real-time PCR. A reduction in the EPO receptor mRNA expression with infection was detected with or without EPO, suggesting an inhibition of EPO receptor expression in \( T. congolense \) infection (Figure 6). Down-regulation of EPO receptor expression seems to impact the proliferation, differentiation, and maturation of the erythroid precursors and leads to inadequate reticulocytosis in the infected animals. However, these mechanisms have yet to be clarified in detail.

The parasitemia of \( T. congolense \)-infected cattle from two breeds, N'Dama and Boran, has not been shown to be significantly different. As shown in Figure 2B, in the chronic phase of infection, the surviving animals with EPO treatment exhibited severe parasitemia with several distinct peaks. Taken together, elevation of parasitemia in the chronic phase of trypanosoma infection does not seem to be related to lethality, at least in the EPO-treated animals. It has been reported that the levels of interferon (IFN)-\( \gamma \) in the kidney and interleukin (IL)-1\( \alpha \), IL-1\( \beta \), and IFN-\( \gamma \) in the bone marrow of \( T. congolense \)-infected Boran are higher than N'Dama. Studies on the pathogenesis of ACD have identified inflammatory cytokines, such as TNF-\( \alpha \), IFN-\( \gamma \), and IL-1, which may be mediators of ACD through their inhibition of the EPO effect on erythroid progenitors. In addition, TNF-\( \alpha \) is thought to contribute to ACD by blocking iron release from the mononuclear phagocytic system, thus depriving the bone marrow of the iron essential to erythropoiesis.

High expression of inflammatory cytokines such as IL-1\( \alpha \), IL-1\( \beta \), and IFN-\( \gamma \) might lead to inadequate erythropoiesis during the course of the disease, especially the acute phase, of trypanosoma infection.

Because pentamidine administration did not improve hematocrit values in infected animals in our experiment (Figure 4A), the effect of EPO and pentamidine on the \( T. congolense \) infection seems to be directed by different mechanisms. Thus, a combination of EPO and pentamidine might have significant potential for the treatment of \( T. congolense \) infection.

The exogenous administration of EPO dramatically improved lethal trypanosome infection in mice. Because lower doses (1,000 U/kg) of EPO showed similar survival by 30 days after infection (data not shown), further experiments would be required to determine a much more effective regimen including dose and treatment period of the EPO. This study has also led to a mouse model system with potential use in analyzing and understanding the pathogenesis of virulent trypanosoma infection in animals, including humans.

References


