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Effect of Recombinant Human Erythropoietin on New Anaemic Model Rats Induced by Gentamicin

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Abstract—The effects of recombinant human erythropoietin (r-HuEPO) on haematological parameters were studied in rats in which uraemia and anaemia had been induced by gentamicin, an aminoglycoside antibiotic and a nephrotoxic agent. After the occurrence of slight polycythaemia, the red blood cell count, haematocrit and haemoglobin concentration decreased by 20–30% compared with those of the control (saline-injected) rats. At the end of gentamicin treatment, the endogenous serum EPO level had decreased to about 40% compared with that of control rats. Gentamicin-treated rats showed marked elevation of blood urea nitrogen, extensive tubular necrosis in the kidney and haemosiderin deposition in the spleen. In the osmotic fragility test, the fragility of erythrocytes significantly increased compared with that of control rats. These findings indicate that the anaemia induced by gentamicin is due not only to a deficiency of EPO but also to an enhancement of fragility of erythrocytes in an azotaemic environment. The administration of r-HuEPO during anaemia markedly increased red blood cell count, haematocrit and haemoglobin concentration. It is suggested that a gentamicin-treated rat is a useful and convenient anaemic model and r-HuEPO is useful for treatment of anaemia in acute renal failure.

Gentamicin, an aminoglycoside antibiotic, produces acute renal failure in man (Wilfert et al 1971; Kahn & Stein 1972; Bennett et al 1980) and experimental animals (Cohen et al 1975; Kahn et al 1980; Rougemont et al 1981; Heller 1984), associated with polyuria, decreased urinary osmolality and the elevation of blood urea nitrogen (BUN) and creatinine. Histological studies have suggested that gentamicin causes acute proximal tubular necrosis in the renal cortex and cast formation in the medulla (Kosek et al 1974; Luft et al 1975; Sugarman et al 1983). However, little is known about the effects of gentamicin on haematological parameters.

Erythropoietin (EPO), a sialyglycoprotein hormone produced mainly in the kidney, regulates the production of red blood cells in mammals (Fisher 1983). The renal peritubular cells are thought to be the primary site of production of EPO by in-situ hybridization (Lacombe et al 1988; Koury et al 1988). In contrast, it has been reported that the regulation of EPO production is related to proximal tubular function (Eckardt et al 1989). Recently, recombinant human EPO (r-HuEPO) (Lin et al 1985) has been highlighted for the clinical treatment of anaemia associated with renal failure. The purpose of the present study was to examine whether anaemia is caused by endogenous EPO deficiency following chronic administration of gentamicin in rats. The effect of r-HuEPO on anaemic animals was also studied.

Materials and Methods

General

Seven week-old, male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), initially 240–280 g, received gentamicin sulphate (Sigma, 100 mg kg^{-1} day^{-1} s.c.) for 17 days. Intact animals given 0·9% NaCl (saline) (1 mL kg^{-1} day^{-1} s.c.) for 17 days were used as controls. Six days later rats received highly purified r-HuEPO (60 or 200 units kg^{-1} day^{-1}, i.v., for 5 days) expressed in Chinese hamster ovary cells (Lin et al 1985) in our laboratory. A 350 μL sample of blood was collected from the tail artery at intervals of 3 or 4 days throughout these studies. The red blood cell (RBC) count, haematocrit and haemoglobin concentration were determined with a blood cell counter (E-2000, Sysmex), and BUN was measured using a commercial test kit (Wako Pure Chemical Industries Ltd, Japan). The smears of the blood cells stained with New Methylene Blue were used for the determination of reticulocyte count. In another experiment, animals were killed with ether the day after the final dose of gentamicin was given. The osmotic fragility test, measurement of blood volumes, radioimmunoassay for serum EPO, and histological studies were as follows.

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Osmotic fragility test

Osmotic resistance to hypotonic pressure was examined according to the method of Parpart et al (1947). Fresh blood or blood incubated for 24 h at 37°C (20 μL) was added to 2 mL of NaCl solution (0·1–1·2%) and left standing for 30 min at room temperature (20°C). The optical density of the supernatant separated by centrifugation was measured at 540 nm. The haemolysis rate (%) was represented as the percentage of total haemolysis induced by 0·1% NaCl solution.

Measurement of blood volume

Blood volume was measured by the dilution method using Evans Blue. Ten minutes after the injection of 0·1% Evans Blue (0·3 mL/animal i.v.), the serum was diluted 10 times with 20 times diluted normal rat serum and its optical density was measured at 610 nm.

Radioimmunoassay for serum EPO level

The endogenous circulating EPO levels of gentamicin-treated rats were compared with those of controls. The crude

rat EPO standard was derived from 3 week-old male Sprague-Dawley rats. Rats were injected with $CoCl_3$ (10 μmol/animal s.c.) and subsequently subjected to hypobaric hypoxia (0·4 atm, for 24 h). The pooled serum EPO level determined by the polycythaemic mice bioassay was 5·9 units mL^{-1}. Radioimmunoassay was by the procedure described by Egrie (1987) with some modifications. Briefly, test samples were incubated at 4°C, for 45–51 h with rabbit antiserum to r-HuEPO at a final dilution of 1:200000 and bound and free r-HuEPO labelled with ^{125}I using iodogen (Pierce Chemical Company) were separated with Tachisorb (goat anti-rabbit IgG conjugated to *Staphylococcus aureus* cells, Calbiochem, CA). Serial dilutions of test samples were checked for parallelism with the standard. The sensitivity of the assay was about 3 m units mL^{-1}.

Histological studies

The kidneys and the spleen were rapidly removed and fixed in buffered 10% formalin and then embedded in paraffin. Sections of the kidney and the spleen were stained with haematoxylin-eosin and Berlin Blue, respectively.

Results

There were no obvious differences between gentamicin-treated and control rats in RBC count, haematocrit and haemoglobin concentration for 10 days from the first dose of gentamicin-sulphate (100 mg kg^{-1} s.c.). However, slight polycythaemia was observed about the time gentamicin was discontinued. During the next month, RBC count, haematocrit, and haemoglobin concentration continued to fall in the treated rats (Fig. 1 A–C) while reticulocyte count in the peripheral blood gradually decreased until it reached zero and then recovered rapidly (Fig. 1D).

The administration of r-HuEPO (60 or 200 units kg^{-1} day^{-1} i.v.) for five consecutive days during anaemia markedly increased RBC count, haematocrit, haemoglobin concentration and reticulocyte count in a dose-dependent manner (Fig. 1). There was a marked increase of haematocrit compared with the increase of RBC count following the administration of r-HuEPO.

The consecutive administration of gentamicin caused marked elevation of BUN and there was no obvious difference in the recovery time course from azotaemia between r-HuEPO-injected rats and vehicle-injected rats (Fig. 2). In the osmotic fragility test performed on the day following the last day of administration of gentamicin, the fragility of erythrocytes of gentamicin-treated rats significantly increased compared with that of control rats in both fresh and incubated blood (Fig. 3). The blood volume and serum EPO level of gentamicin-treated rats decreased to 70 and 40%, respectively, compared with those of control rats (Table 1). Histological studies showed extensive tubular

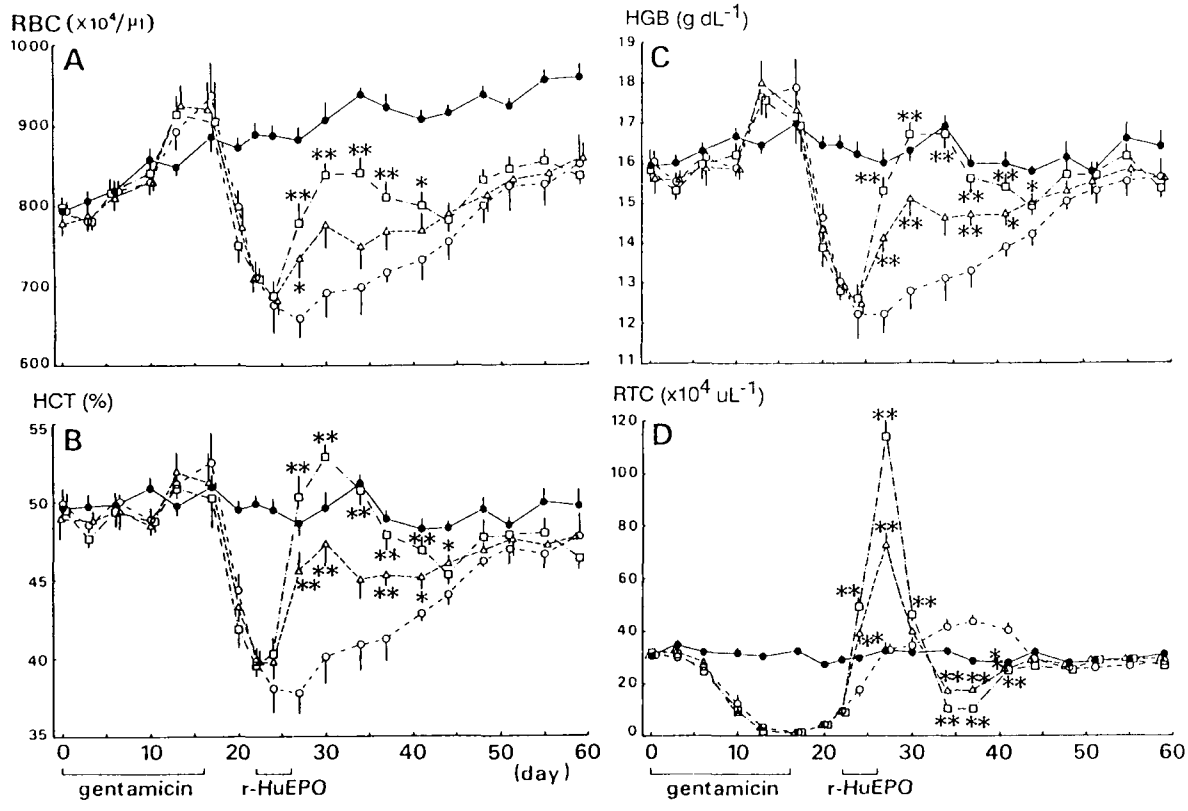

FIG. 1. Effects of r-HuEPO (vehicle 1 mL kg^{-1} day^{-1} i.v.: ○, 60 units kg^{-1} day^{-1} i.v.: △, 200 units kg^{-1} day^{-1} i.v.: □ for 5 days) on red blood cell (RBC) count (A), haematocrit (HCT) (B), haemoglobin (HGB) concentration (C) and reticulocyte (RTC) count (D) in anaemic rats induced by gentamicin-sulphate (100 mg kg^{-1} day^{-1} s.c. for 17 days). (●) Control rats (saline, 1 mL kg^{-1} day^{-1}, s.c., for 17 days). Each point represents the mean ± s.e. of 7 animals. $^*P < 0·05$, $^{**}P < 0·01$ (two-tailed Student's *t*-test) compared with EPO vehicle-injected rats.

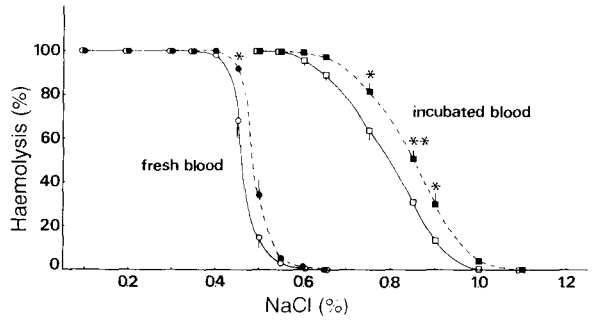

FIG. 2. Blood urea nitrogen (BUN) in gentamicin-treated rats (open symbols) and control rats (●). The doses of r-HuEPO were vehicle (○), 60 units kg^{-1} (△) and 200 units kg^{-1} (□). Each point represents the mean ± s.e. of 7 animals. Where s.e. bars are not shown, they lie within the dimensions of the symbols.

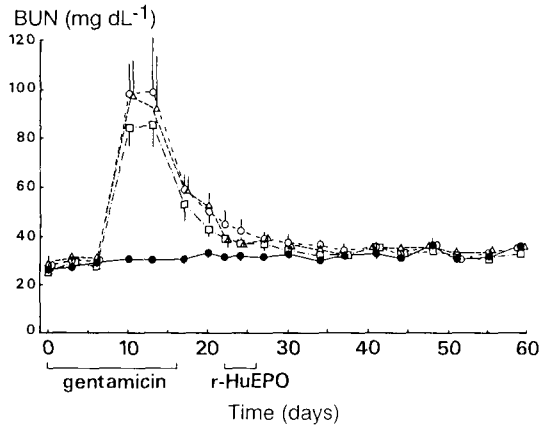

FIG. 3. Osmotic resistance of fresh erythrocytes (○,●) and incubated erythrocytes (□,■) to hypotonic pressure. Erythrocytes were obtained from gentamicin-treated rats (●,■) and control rats (○,□). Each point represents the mean ± s.e. of 4 animals. Where s.e. bars are not shown, they lie within the dimensions of the symbols. $*P<0.05$, $**P<0.01$ (two-tailed Student's t-test) compared with control rats.

Table 1. The blood volume measured by the method of dilution with Evans Blue, and endogenous EPO determined by radioimmunoassay in control rats and gentamicin-treated rats. Each value represents the mean ± s.e. of 4 or 6 animals.

	Control	Gentamicin treated
Blood volume (mL)	20·2 ± 0·9 (n = 4)	13·8 ± 0·3** (n = 4)
EPO level (m units mL^{-1})	33·1 ± 4·2 (n = 6)	13·2 ± 1·4* (n = 6)

$*P<0.05$, $**P<0.01$ (two-tailed Student's t-test) compared with control rats.

necrosis in the kidney and haemosiderin deposition in the spleen of the treated rats (Figs 4, 5).

Discussion

After the occurrence of slight polycythaemia, anaemia was observed in gentamicin-treated rats (Fig. 1). The polycythaemia observed at the time gentamicin treatment ceased is thought to be the result of haemoconcentration related to decreased food and water intake (Heller 1984), or increased urinary volume (Kahn et al 1980; Rougemont et al 1981). The decrease in blood volume in the present study (Table 1) strongly supports the aetiology of the apparent polycythaemia observed in the treated rats. It is possible that the lack of information on whether gentamicin causes anaemia is because of this haemoconcentration during the administration of gentamicin. Therefore, the anaemia observed following the polycythaemia could be related to the recovery from the fall in blood volume following withdrawal of gentamicin.

The anaemia induced by gentamicin may be caused by several factors. Firstly, marked deficiency of endogenous EPO level measured by radioimmunoassay (Table 1) is probably the primary etiologic factor. This is supported by the histological observation that renal tubular cells which regulate EPO production (Eckardt et al 1989) showed extensive necrosis (Fig. 4). In addition, decreased plasma EPO levels have also been reported in rats with tubular cell necrosis induced by uranyl nitrate (Giglio et al 1986).

Secondly a shortened erythrocyte life span could be another factor leading to anaemia. It has been reported that long-lasting azotaemia causes haemolysis in patients with chronic renal failure (Joske et al 1956; Loge et al 1958; Shaw 1967). In the present study, the fragility of erythrocytes in gentamicin-treated rats significantly increased compared with that in controls (Fig. 3). This increased fragility of erythrocytes is considered to be due to the uraemic toxins associated with marked elevation of BUN (Fig. 2). Furthermore, extensive haemosiderin deposition in the spleen of gentamicin-treated rats (Fig. 5) supports the shortened life span of erythrocytes in azotaemic conditions.

Thirdly, possible inhibitors of erythropoiesis in the serum of the treated rats may also play a part. The existence of inhibitors in the serum of uraemic, anaemic rabbits (Ohno & Fisher 1977) and patients (Ohno et al 1978) has been demonstrated by studies of in-vitro erythroid colony formation.

Fourthly, there is a possibility that gentamicin itself has a direct toxic effect on progenitor cells in the bone marrow. However, this might be excluded by the observation that decreased white blood cell and platelet counts were not observed in treated rats (data not shown).

Therefore, these aetiological factors leading to anaemia in the treated rats are considered to be sufficient for the aetiology of anaemia in patients with chronic renal failure.

The administration of r-HuEPO during anaemia markedly increased RBC count, haematocrit, haemoglobin concentration and reticulocyte count (Fig. 1). There was marked increase in haematocrit compared with that in RBC count following the administration of r-HuEPO. This may be due to the increased mean corpuscular volume resulting from the appearance (Fig. 1 D) of a slightly larger reticulocyte than that of matured erythrocytes. It has been reported that the response to EPO in chronic renal failure patients (Van Dyke et al 1963) and in nephrectomized animals (Reissmann et al 1960; Bozzini et al 1966) is less than in normal controls. However, it has also been suggested that an azotaemic environment induced by nephrectomy does not decrease the response to EPO (Kurtides et al 1965; Anagnostou et al 1977). In the present study, there was a rapid and marked response to r-HuEPO, which was independent of the

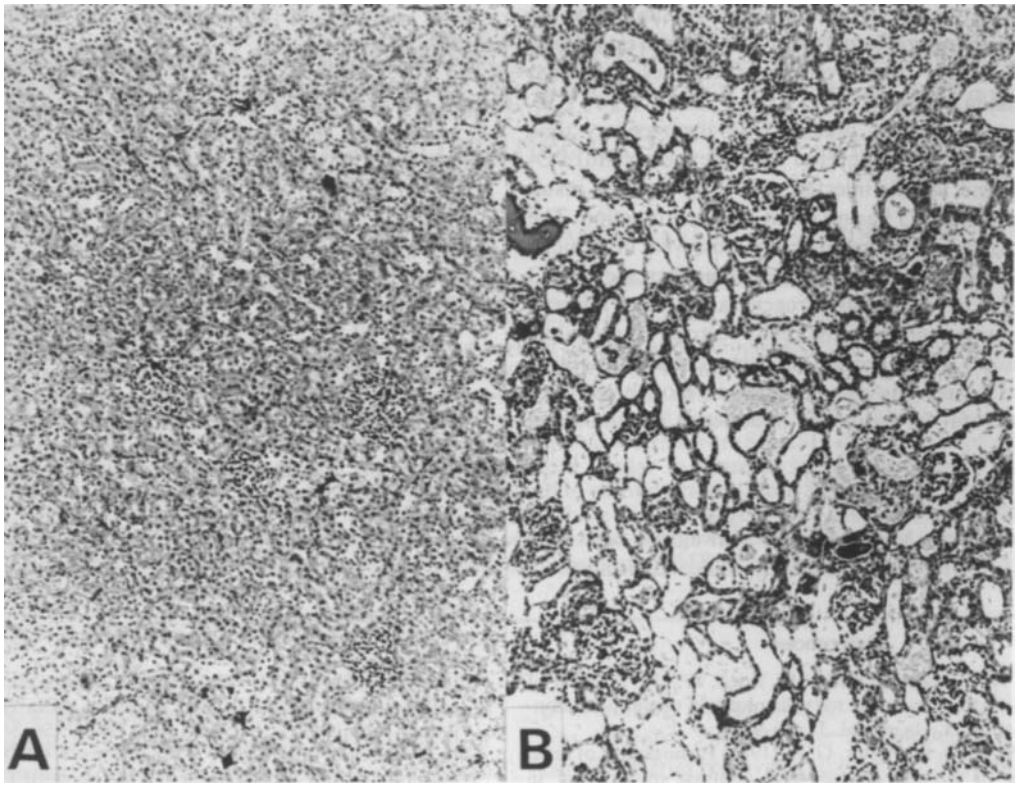

FIG. 4. Photomicrograph of the renal cortex in control (A) and in gentamicin-treated rats (B). Extensive tubular necrosis is present in treated rats (B) Haematoxylin-eosin (× 100).

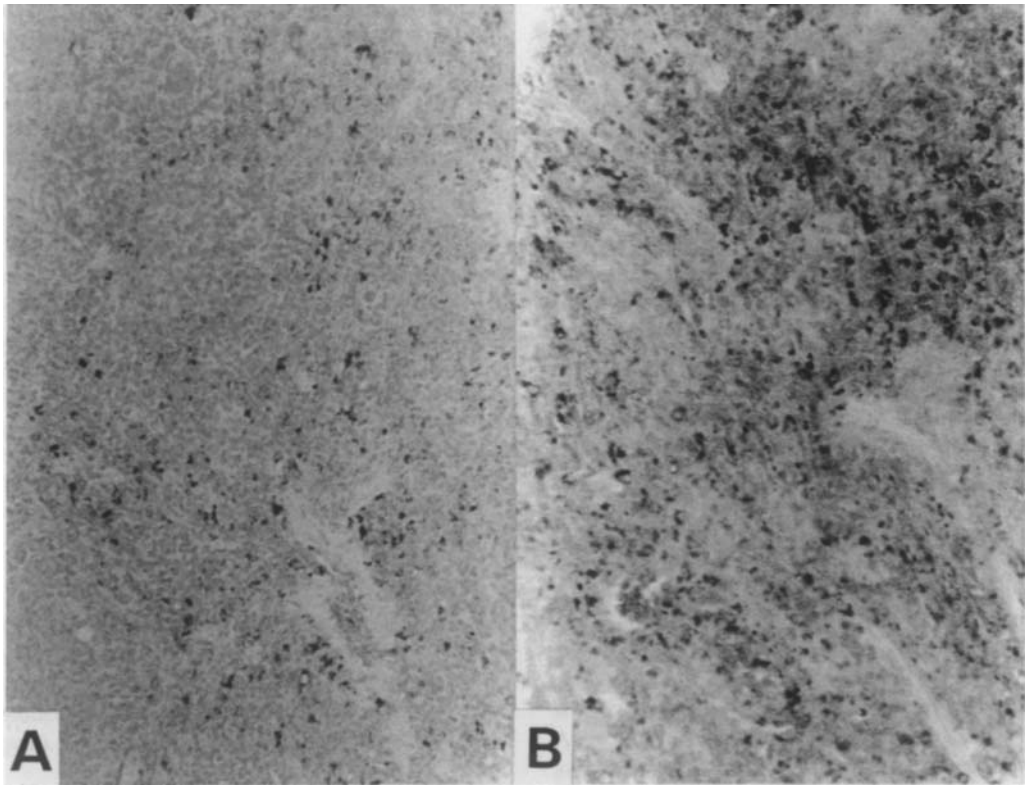

FIG. 5. Photomicrograph of the spleen in control rats (A) and in treated rats (B). Excessive deposition of haemosiderin is seen in gentamycin-treated rats (B). Berlin Blue (× 100).

azotaemic environment. This response was similar to the results obtained in a preliminary test with normal rats. These results are consistent with the reports of Kurtides et al (1965) and Anagnostou et al (1977).

There was no obvious difference in the recovery time course from the increased level of BUN between r-HuEPO-injected rats and vehicle-injected rats (Fig. 2). This result suggests that r-HuEPO has no effect on renal function. The recovery time course from anaemia observed in vehicle-injected rats is considered to be related to an increase in EPO production due to renal tubular regeneration, since it is well known that gentamicin-induced renal necrosis is reversible.

In conclusion, it is suggested that the gentamicin-treated rat is a useful and convenient anaemic model and that r-HuEPO is useful for treatment of anaemia in acute renal failure.

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