The biological properties of endotoxin-free human erythropoietin

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(Received 10 November 1980/Accepted 19 March 1981)

The biological effects formerly attributed to erythropoietin were generally observed using impure preparations of the hormone containing, among the impurities, bacterial endotoxin, a contaminant that has recently been shown to affect the haemopoietic system. Erythropoietin, purified to apparent homogeneity and freed of trace contamination by endotoxin (1) promotes erythroid burst formation from mouse marrow cells in semi-solid culture medium, (2) stimulates haemoglobin synthesis in rat marrow cell cultures, (3) stimulates transcription in mouse and rat bone marrow cell cultures and (4) causes the formation of the cytoplasmic mediator protein in rat marrow cells, which enhances transcription in isolated nuclei.

Most of the published data on the effects of erythropoietin have been obtained from experiments using preparations that were far from pure, and in those few reports in which erythropoietin, purified to apparent homogeneity, was used, no attention was paid to the possible effects of contaminants that may affect the haemopoietic system. The action of these contaminants may make the interpretation of erythropoietin action ambiguous. Recently it was reported that bacterial endotoxin was a contaminant of erythropoietin preparations and did itself have an effect on the haemopoietic system by decreasing the number of erythroid bursts and colonies (Udapa & Reissmann, 1977; Zuckerman *et al.*, 1979) as well as by decreasing the ^{59}Fe incorporation into red cells (Schade & Fried, 1976). Endotoxin caused an increase in cyclic GMP levels in foetal liver cells, an effect that had previously been attributed to erythropoietin (Graber *et al.*, 1979). It was therefore important to determine whether erythropoietin was, in fact, responsible for the effects on bone marrow cells formerly ascribed to it. We report here that human erythropoietin, purified to apparent homogeneity and freed of contaminating endotoxin (*a*) stimulates transcription in mouse and rat bone marrow cell cultures, (*b*) causes the formation of a marrow cytoplasmic factor in rat marrow cells, which enhances transcription in isolated nuclei, (*c*) stimulates haemoglobin synthesis in rat marrow cell cultures and (*d*) promotes erythroid burst formation from mouse marrow cells in semi-solid culture medium. We have also shown that erythropoietin has no effect on the specific radioactivity of the intracellular UTP pool when marrow cells are labelled with [^3H]uridine.

Experimental

Materials

The *Limulus* amoebocyte lysate test for endotoxin, E-Toxa-Clean and phenylmethanesulphonyl fluoride, nucleotide triphosphates, phosphoenolpyruvate and pyruvate kinase and L-cysteine hydrochloride were obtained from Sigma Chemical Co. The radioactively labelled compounds $^{59}\text{FeCl}_3$, [^3H]uridine (sp. radioactivity 26 Ci/mmol) and [^3H]UTP (sp. radioactivity 10–15 Ci/mmol) were supplied by Amersham Radiochemical Co. Nonidet P40 came from Bethesda Research Laboratories. Pyrogen-free water was purchased from Travenol Co. Pyrogen-free human serum albumin came as a 5% solution from Cutter Laboratories. Foetal calf serum came from Flow Laboratories and new-born calf serum and N.C.T.C.-135 were bought from Grand Island Biological Company. Rat serum was prepared in this laboratory from Sprague–Dawley rats.

Methods

The procedure used to remove endotoxin from erythropoietin preparations was a modification of that described by Dr. R. Shaddock (personal communication). All glassware was soaked overnight in a 1% solution of the detergent E-Toxa-Clean, rinsed thoroughly with water followed by a final rinse with pyrogen-free sterile water. The glassware was autoclaved for 1 h and then heated at 175°C for 3 h. Purified human urinary erythropoietin (1 ml) (Miyake *et al.*, 1977) was layered on a 1.5 ml cushion of pyrogen-free albumin in 3 ml quartz centrifuge tubes, and centrifuged in a

Beckman SW 50L rotor at 140000g for 19 h. The erythropoietin diluent 0.15 M-NaCl/0.1% bovine serum albumin/10 mM-CaCl₂ was treated in an identical manner, and used for the control experiments. The upper 1.0 ml containing 70–100% of the erythropoietin was removed after centrifugation and tested for endotoxin using the *Limulus* amoebocyte lysate procedure (Yin *et al.*, 1972). There was no detectable endotoxin found.

All bone marrow cultures were prepared from femora and tibiae of male Long Evans rats (approx. 3 months old) or from BDF₁ female mice (approx. 3 months old). Those experiments measuring ⁵⁹Fe incorporation into haemoglobin were performed as described by Goldwasser *et al.* (1975). Rat marrow cells were seeded at a concentration of 15 × 10⁶ cells/ml in medium containing 65% N.C.T.C.-135 [with 30 mM-Mops(4-morpholinepropanesulphonic acid), 1.65 mM-cysteine/HCl, 30 mM-NaHCO₃ and 1 μl of β-mercaptoethanol/100 ml], 30% foetal calf serum (with prior heating at 56°C for 30 min), 5% rat serum [with 73 μM-Fe(NO₃)₃] and 50 μg of gentamycin/ml (Schering Corp.). Six replicates per experiment of 0.2 ml cell suspensions for each erythropoietin concentration were pipetted into a 24-well Linbro tray (Linbro Scientific Co.) and incubated at 37°C in CO₂/air (3:97).

Erythroid burst forming cells (BFU-E assay).

Mouse marrow cells were seeded at 2 × 10⁵ nucleated cells/ml in methylcellulose cultures as described by Iscove & Sieber (1975). The cultures were incubated at 37°C in CO₂/air (3:97) and benzidine-positive bursts were counted after 8 days.

Marrow cell transcription. Each experiment consisted of five replicates per time point (0.3 ml/replicate) of bone marrow cell cultures from Long Evans rats or BDF₁ mice. A minimum of three experiments were done with each species. The cells were seeded in 24-well Linbro plates at a concentration of 30 × 10⁶ nucleated cells/ml in the same medium used for the ⁵⁹Fe assays and pre-incubated for approx. 2 h at 37°C in CO₂/air (1:19). Erythropoietin (0.2 units/ml of culture medium) or the same volume of diluent was added and each culture was incubated for increasing time intervals, after which [³H]uridine was added at a concentration of 2 μCi/ml of culture medium. After an additional 20 min of incubation in the presence of [³H]uridine, the cultures were transferred to test tubes and the culture wells were washed twice with cold phosphate-buffered saline (0.135 M-NaCl/5 mM-KCl/10 mM-potassium phosphate, pH 7.4). The phosphate-buffered-saline washes were pooled with the cells, which were then collected by centrifugation at 1000g for 10 min at 4°C and lysed with cold 10% trichloroacetic acid/10 mM-sodium pyrophosphate. The acid-insoluble precipitate was washed

twice with this same solution, dissolved in 0.4 ml of 77% formic acid and heated at 100°C for 10 min. For the mouse marrow samples a 120 μl portion was counted for radioactivity in Aquasol and the DNA content was determined on a 240 μl portion by the method of Burton (1956). For the rat marrow system an 80 μl portion was counted for radioactivity and 280 μl was used for the DNA determination.

Marrow cytoplasmic factor preparation and assay. Rat marrow cell cultures in a medium consisting of 90% N.C.T.C.-135 (with the same additions described above) and 10% newborn-calf serum were seeded at a concentration of 30 × 10⁶ nucleated cells/ml and pre-incubated for 46 h at 37°C in a CO₂/air (1:19). Erythropoietin (0.2 units/ml of culture) or an identical volume of diluent was then added and the cultures were incubated for one additional hour. The cells were transferred to tubes by washing with phosphate-buffered saline and centrifuged as described above. The cell pellets were resuspended in cold buffer [0.8 M-sucrose/0.1 M-Tris (pH 8.0)/8 mM-MgCl₂/4 mM-CaCl₂/0.8 mM-phenylmethanesulphonyl fluoride/0.25% Nonidet P40] and kept on ice for 20 min. The nuclei and unlysed cells were then centrifuged at 1000g for 15 min; the supernatant from the erythropoietin-treated cells was used as the crude marrow-cytoplasmic-factor preparation and the supernatant from cells treated with the diluent as control.

A two-step transcriptional assay with marrow nuclei was performed as previously described (Inana, 1977) with the following modifications. A 150 μl portion of nuclei was incubated for 25 min at 25°C with 150 μl of marrow-cytoplasmic factor or control cytosol, both previously adjusted to a protein concentration of 12 mg/ml using the A₂₈₀/A₂₅₀ ratio to estimate protein concentration (Layne, 1957). The nuclei were then collected by centrifugation at 1000g for 10 min and resuspended in the transcriptional assay solution containing [³H]UTP (10 μCi/ml of reaction solution). Five replicate tubes per assay point were incubated at 25°C for increasing lengths of time before the RNA was precipitated.

Specific radioactivity of UTP pool. Rat marrow cell cultures containing 30 × 10⁶ nucleated cells/ml were pre-incubated for 30 min at 37°C in CO₂/air (1:19), after which 0.2 unit of endotoxin-free erythropoietin/ml or an equal volume of diluent (0.1% bovine serum albumin/10 mM-CaCl₂/0.15 M-NaCl) was added to the cultures. The cultures were incubated for an additional 5 h, then [³H]uridine (20 μCi/ml of culture) was added and the cells were incubated for an additional 30 min. The acid-soluble fraction was prepared by precipitation at a final concentration of 0.5 M-HClO₄, and centrifuging the precipitate at 10800g for 10 min. A 0.95 ml portion

of supernatant was neutralized with 5 M-KOH and the KClO_4 was removed by centrifugation at 10800g for 10 min. Measurement of UTP concentration and radioactivity of the supernatant were performed on each sample by Dr. Kyle Webster, Walter Reed Army Hospital, Washington, DC, U.S.A., using an ion-exchange high-pressure liquid chromatography system developed by him.

Results

Haemoglobin synthesis by rat marrow cells was measured as a function of endotoxin-free erythropoietin concentration. A linear dose-response curve was obtained from the endotoxin-free preparation (Fig. 1), which was not statistically different from the curve obtained from the erythropoietin sample containing endotoxin.

The effects of erythropoietin on transcription *in vitro* by marrow cells reported previously from this laboratory were found using rat marrow cells (Gross & Goldwasser, 1969), yet the mouse is the preferred system to study clonal colony development. To ascertain whether or not the early molecular effects of erythropoietin in mouse and rat marrow cells were similar, it was important to determine whether pure erythropoietin, free of endotoxin, could stimulate transcription in mouse marrow cell cultures. Mouse marrow cells incubated for 20 min with [^3H]uridine without preincubation in one experiment incorporated 5120 ± 270 d.p.m./mg of DNA with no erythropoietin added and 6870 ± 340 d.p.m./mg of DNA with 0.2 units of endotoxin-free erythropoietin/ml, a stimulation of 34%. In an identical experiment, rat

cells showed a 20% increase (1280 ± 90 and 1530 ± 95 d.p.m./mg of DNA) in specific radioactivity of RNA. Both of these differences were statistically significant at the $P < 0.05$ level by *t* test. In another experiment at a later time of incubation, when there is a decreased rate of transcription, the effect of endotoxin-free erythropoietin was somewhat greater. The increase in the 20 min incorporation of uridine was 78% (1480 ± 30 and 2630 ± 630) at 4 h for mouse cells and 54% (1160 ± 100 and 1790 ± 230) for rat cells at 5 h. These differences were also statistically significant at the $P < 0.05$ level.

Cellular transcription was measured by the amount of labelled UMP incorporated into acid-insoluble RNA. The increased label found in this acid-insoluble fraction of erythropoietin-treated samples could have resulted from an effect of erythropoietin on increased rate of RNA synthesis, on decreased rate of RNA degradation, and/or on an effect on the UTP pool resulting in a pool of greater specific radioactivity. To determine that the measured erythropoietin effect was not simply due to a change in the specific radioactivity of the RNA precursor pool this parameter was measured in the erythropoietin-treated and control rat marrow cells. The specific radioactivity of UTP in the erythropoietin-treated cells (320 c.p.m./nmol) was the same as that in control cells (330 c.p.m./nmol). No significant difference was observed in the pool size of the two samples, with 12.4 nmol of UTP/ml of supernatant measured in the erythropoietin-treated cells and 10.1 nmol of UTP/ml of supernatant measured in control cells.

We have proposed that the erythropoietin effect on the stimulation of nuclear transcription by marrow cells is mediated via an intracellular protein factor (Chang & Goldwasser, 1973; Goldwasser & Inana, 1978). Since the erythropoietin preparation previously used to study this mediator factor was impure, it was crucial to determine whether these results could be duplicated with pure endotoxin-free erythropoietin. As shown in Fig. 2, endotoxin-free erythropoietin induces the formation of this mediator factor activity in rat marrow cells, which, when assayed with isolated rat marrow nuclei, stimulates transcription up to 200%, a stimulation considerably greater than that previously reported.

Most of the clonal colony assays previously reported have been performed using impure erythropoietin preparations. An erythropoietin preparation, free of endotoxin and purified to apparent homogeneity, was used in the erythroid burst assay to determine whether erythropoietin is the agent that induces erythroid burst development. The results, 20 ± 4 erythroid burst forming units observed in cultures containing purified erythropoietin contaminated with endotoxin and 24 ± 5 erythroid

Fig. 1. Dose response of rat marrow cells to endotoxin-free purified erythropoietin

Rat marrow cell cultures were incubated for 24 h at 37°C , in CO_2/air (1:19) with increasing amounts of erythropoietin (O) or an endotoxin-free preparation of the same erythropoietin (■). Subsequently, ^{59}Fe was added to the cultures and the cells were incubated an additional 5 h. The haemoglobin was then precipitated, extracted and the amount of label incorporated was determined. The points are means and the vertical bars indicate standard deviations calculated from six replicate samples.

Fig. 2. Effect of endotoxin-free purified erythropoietin on the formation of marrow cytoplasmic factor

The crude preparation of the intracellular mediator factor was isolated from rat marrow cells incubated with endotoxin-free purified erythropoietin and the control preparation was made from marrow cells incubated with the erythropoietin diluent. After prior incubation with either marrow cytoplasmic factor (O) or the control cytosol (■) isolated marrow cell nuclei were incubated with [3 H]UTP for increasing lengths of time. The label incorporated into the acid-insoluble fraction was determined. The DNA content was measured for each replicate sample and the results are expressed as d.p.m./μg of DNA. The points are means and the vertical bars indicate standard deviations calculated from six replicate samples.

burst forming units measured in cultures containing purified erythropoietin freed of endotoxin, indicate no difference between erythropoietin containing endotoxin and endotoxin-free erythropoietin with respect to the effect on the number of bursts. The number of bursts scored were somewhat low but within the range generally found in this laboratory. Cultures without exogenous erythropoietin do not form 8-day erythroid bursts.

Discussion

Impure preparations of erythropoietin have been reported to stimulate cellular transcription in marrow (Gross & Goldwasser, 1969), in foetal liver (Paul & Hunter, 1969; Djaldetti *et al.*, 1972) and in polycythaemic spleen (Fujioka, 1966; Piantadosi *et*

al., 1976). Also, these impure preparations of erythropoietin have been reported to stimulate haemoglobin synthesis in marrow (Krantz *et al.*, 1963) and in foetal liver (Marks & Rifkind, 1972), marrow cytoplasmic factor production (Chang & Goldwasser, 1973; Goldwasser & Inana, 1978) and erythroid burst formation (Iscoe & Sieber, 1975). Any effects possibly contributed by the contaminants, however, have largely been ignored, thereby leaving the biological effects of erythropoietin on marrow cells still uncertain. Only recently has one trace contaminant been shown to affect the haemopoietic system. Endotoxin, a contaminant of many erythropoietin preparations, has been shown to suppress marrow cell response to erythropoietin *in vivo* and *in vitro* by reducing the number of erythroid burst-forming and colony-forming units (Udapa & Reissmann, 1977; Zuckerman *et al.*, 1979) and to decrease the ^{59}Fe incorporated into red cells (Schade & Fried, 1976). Endotoxin has also been shown to cause the increase in cyclic GMP concentration in rat foetal liver cells, an effect formerly ascribed to erythropoietin (Graber *et al.*, 1979). Since endotoxin is just one of the many contaminants in the erythropoietin preparation used in these studies, it was possible that the other contaminants could also have contributed to the observed effects previously attributed to erythropoietin.

We have now shown that apparently homogeneous erythropoietin, free of endotoxin, retains the biological activity, measured by haemoglobin synthesis and burst formation, originally present in the preparation containing endotoxin. The erythropoietin effects on stimulation of whole-cell transcription and on the formation of marrow cytoplasmic factor activity, as determined by transcription in isolated nuclei, have now been shown to be due to endotoxin-free hormone.

Previous work has shown that label found in the acid-insoluble precipitate from control and erythropoietin-treated cells incubated with [3 H]uridine is ribonuclease-sensitive (Bedard & Goldwasser, 1976). The conclusion that erythropoietin stimulated the rate of transcription was derived from experiments in which the cells were prelabelled with uridine (Gross & Goldwasser, 1969). However, any possible effect of erythropoietin on the specific radioactivity of the intracellular UTP pool was not measured. Direct measurement of the UTP pool showed no difference in the specific radioactivity of erythropoietin-treated and control marrow cells, substantiating the conclusion that erythropoietin stimulates the rate of transcription.

This research was done with partial support from grants HL 21676 and CA 18375 from the National Institutes of Health.

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