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ORIGINAL ARTICLE

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R-h-erythropoietin counteracts the inhibition of in vitro erythropoiesis by tumour necrosis factor alpha in patients with rheumatoid arthritis

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Abstract Anaemia of chronic disease (ACD) is a common extra-articular manifestation of rheumatoid arthritis (RA). Tumour necrosis factor alpha (TNFα) plays an important role in the development of ACD. The objective of the present study was to assess inhibition of in vitro colony-forming unit erythrocyte (CFUe) and blast-forming unit erythrocyte (BFUe) growth by TNFα and to examine whether this suppression could be counteracted by adding increasing concentrations of recombinant human erythropoietin (EPO) (r-h-EPO) to bone marrow cultures of RA patients with ACD and without anaemia (controls). Bone marrow cells of RA patients with ACD and control patients were cultured. The cultures were incubated with increasing concentrations of r-h-EPO (0.25; 0.5; 1; 2 U/ml), each in combination with increasing quantities of TFNα (0; 50; 100; 200; 400 U/ml). CFUe and BFUe were assessed after 7 and 14 days, respectively. Dose-dependent inhibition of BFUe and CFUe by increasing concentrations of TNFα was observed in ACD and controls. Regarding CFUe (ACD patients) incubated with 0.25 U/ml EPO, 50 U/ml TNFα caused 28% suppression compared to cultures without TNFα. Increasing the concentration of r-h-EPO from 0.25 U/ml to 2 U/ml completely restored the number of CFUe. A similar pattern was observed in BFUe growth in both groups. These data demonstrated the suppressive effects of TNFα on erythropoiesis in vitro and that the suppressed erythropoiesis could be partly corrected by the addition of excess r-h-EPO to the cultures. No significant differences were observed between ACD and control RA patients. This in vitro model may help explain the clinical response to r-h-EPO therapy as documented in RA patients with ACD.

Key words TNF · Anaemia of chronic disease · Erythroid progenitors · Erythropoietin · Rheumatoid arthritis

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Introduction

Anaemia of chronic disease (ACD) is a common extra-articular manifestation of active rheumatoid arthritis (RA). Cytokines such as interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNFα) are immunoregulatory factors and may be involved in the pathogenesis of rheumatic diseases and ACD [1–4]. ACD is associated with inflammatory conditions associated with macrophage activation. Activated macrophages produce a number of cytokines including IL-1, TNFα, interferon alpha and gamma (IFNα, IFNγ), which may have an affect on erythropoiesis [5, 6], thereby participating in the development of ACD. Previous in vitro studies have shown the inhibition of CFUe and BFUe by TNFα [7–11]. Whether these effects of TNFα on erythroid progenitors are direct [8] or whether the presence of other cells is required [10, 11] is not entirely clear. In addition, chronic TNFα exposure has been shown to suppress erythropoiesis in vivo [12, 13]. Nude mice inoculated with Chinese hamster ovary (CHO) cells expressing the human TNFα gene developed a hypoferraemic, hypoproliferative anaemia with normal iron stores and decreased numbers of bone marrow and splenic CFUe and BFUe. These data suggest that chronic TNFα exposure in vivo may be involved in the development of ACD. Serum TNFα concentration has been shown to be elevated in RA patients with ACD and correlates well with RA disease parameters [14]. Based on the inverse correlation of serum TNFα and haemoglobin in the above-mentioned study [14] and in a group of HIV patients [15], it may be argued that TNFα plays a specific role in ACD.

Low plasma erythropoietin (EPO) levels in relation to the degree of anaemia in patients with acute or chronic tion, inflammation or malignancy despite a normal renal function [16–20]. Therefore, it is postulated that ACD patients are characterised by a relatively low EPO production. Decreased EPO sensitivity progenitors is postulated in RA and ACD [19]. ACD can be corrected with recombinant human EPO (r-h-EPO) therapy in patients with RA [20–25]. It has already been demonstrated that anaemia in mice caused exclusively by chronic TNF exposure can be corrected by administration of exogenous EPO [25]. Therefore, one may postulate that the beneficial effects of EPO on ACD in RA patients can be explained, at least to some extent, by the ability of EPO to conteract TNF-mediated suppression of erythropoiesis. Therefore, we studied whether the suppressive effects of TNFα on erythropoiesis in vitro could be corrected with increasing r-h-EPO concentrations. We were also interested in possible differences between TNFα-inhibited CFUe and BFUe colony growth and whether these effects would differ between RA patients with ACD and RA patients without anaemia.

Patients and methods

Preparation of cell suspension

Bone marrow (BM) of eight RA patients with ACD (group 1) and eight RA patients without anaemia (control patients; group 2) was aspirated from the posterior iliac crest after the patients had given informed consent. The marrow was collected in Hank's balanced solution (HBBS) with heparin, diluted in HBBS, and mononuclear cells were separated over Ficoll-Isopaque (1.077 g/cm^2; Nycomed, Oslo, Norway).

Recombinant human cytokines

Recombinant human TNFα (1 U/ml is 16 pg/ml) was provided by Boehringer Institute (Vienna, Austria). The concentration of TNFα was a indicated in the figures. R-h-EPO (Boehringer, Mannheim) was used as shown in Fig. 1.

Colony culture assay

Progenitor cells (0.5–1 × 10^5 cells/ml) of eight ACD patients and eight control patients were cultured in a mixture of Iscove's modified Dulbecco's medium (IMDM), 1.1% methylcellulose, 30% fetal calf serum (FCS), bovine serum albumin (BSA), transferrin, lecithin, sodium selenite and 2-mercaptoethanol in a humidified atmosphere of 5% CO_2 in air at 37 °C as reported previously [25]. Increasing concentrations of r-h-EPO (0.25; 0.5; 1; 2 U/ml), each in combination with increasing concentrations of TNFα (0; 50; 100; 200; 400 U/ml) were added to the cultures. CFUe and BFUe were scored after 7 and 14 days, respectively.

Statistics

The Wilcoxon signed-rank test was used to calculate statistical differences between the number of CFUe or BFUe under different conditions of incubation within one group. To established differences in CFUe or BFUe between the two groups, the two-sample Wilcoxon rank sum test was used.

Results

Inhibition of erythropoiesis by TNFα in vitro

TNFα suppressed CFUe and BFUe growth in a dose-dependent manner (Tables 1 and 2; Fig. 1). The same inhibition occurred in both groups.

Effect of r-h-EPO and CFUe and BFUe

After the addition of increasing concentrations of r-h-EPO (0.25 U/ml up to 2 U/ml) the number of CFUe and BFUe increased in a dose-dependent manner. In cultures

Table 1 Effect of erythropoietin (EPO) and tumour necrosis factor alpha (TNFα) on colony forming unit erythrocyte growth. Bone marrow of eight anaemia of chronic disease (ACD) patients and eight controls was incubated with increasing concentrations of EPO each in combination with increasing concentrations of TNFα. The median number of colonies and the range is shown

TNF U/ml	EPO U/ml							
	0.25		0.5		1.0		2.0	
	ACD	Controls	ACD	Controls	ACD	Controls	ACD	Controls
0	257 (32–455)	241 (42–425)	295* (10–508)	211 (22–590)	302* (25–522)	293* (30–525)	327* (10–500)	313* (30–867)
50	209** (15–290)	206** (12–386)	284* (15–681)	240 (15–475)	285* (7–543)	289* (15–400)	293* (15–538)	333* (35–502)
100	183** (7–297)	196** (7–290)	222*,** (0–451)	222*,** (35–475)	286* (2–544)	275* (32–350)	286* (2–370)	273* (32–347)
200	157** (5–235)	114** (17–305)	247*,** (2–392)	198* (15–280)	256* (7–362)	226* (45–275)	272*,** (10–399)	238 (32–322)
400	127** (2–269)	140** (10–195)	216*,** (5–265)	168*,** (15–280)	238* (5–297)	212* (25–387)	237*,** (0–361)	222* (35–322)

* $P < 0.05$ (compared to basic culture where 0.25 U/ml EPO was added without change in TNFα concentration)
** $P < 0.05$ (compared to basic culture where no TNFα was added and no change in EPO concentration)

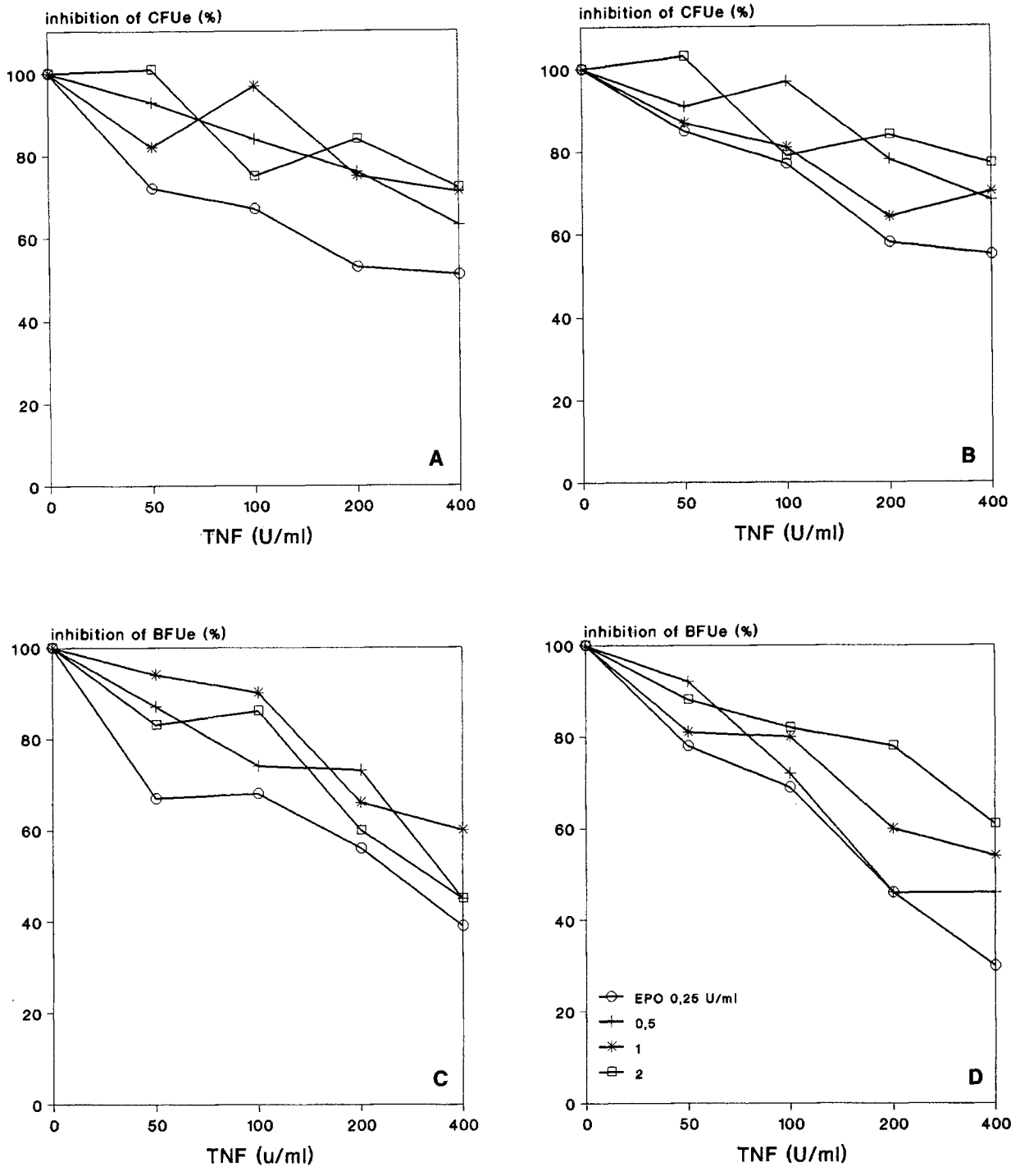

Fig. 1 Effects of varying recombinant human erythropoietin (r-h-EPO) concentrations on inhibition of marrow cell colony forming units erythrocyte (CFUe) and blast forming unit erythrocyte (BFUe) colony formation by tumor necrosis factor alpha (TNFα). Results are expressed as median of eight cultures for each group. CFUe and BFUe colony formation in cultures incubated without TNFα are defined as 100%. **A** CFUe colony formation in anaemia of chronic disease (ACD) patients (group 1); **B** CFUe colony formation ion control patients (group 2); **C** BFUe colony formation in group 1; **D** BFUe colony formation in group 2. Increasing concentrations of TNFα were combined with four different concentration of r-h-EPO: (o) 0.25 U/ml; (|) 0.5 U/ml; (*) 1 U/ml; (□) 2 U/ml

from both groups with 2 U/ml r-h-EPO and no TNFα, we found a significantly higher CFUe score as compared to cultures incubated with 0.25 U/ml r-h-EPO. No significant differences between the groups (Tables 1 and 2; Fig. 1) were demonstrated.

Effect of r-h-EPO on TNFα-suppressed CFUe and BFUe

TNFα suppression was corrected with the addition of excess r-h-EPO as shown in Tables 1 and 2 and Fig. 1. Regarding CFUe (in group 1), incubation with 0.25 U/ml

Table 2 Effect of EPO and TNFα on blast forming unit erythrocyte growth. Bone marrow of eight ACD patients and eight controls was incubated with increasing concentrations of EPO each in combination with increasing concentrations of TNFα. The median number of colonies and the range is shown

TNF U/ml	EPO U/ml							
	0.25		0.5		1.0		2.0	
	ACD	Controls	ACD	Controls	ACD	Controls	ACD	Controls
0	252 (45–300)	163 (75–310)	231* (37–287)	161 (115–340)	265 (22–400)	198* (140–342)	287 (22–482)	207* (130–442)
50	181** (15–220)	140 (52–190)	232* (2–357)	154 (70–257)	264* (22–380)	168*,** (65–302)	233* (22–342)	191*,** (85–292)
100	188*,** (15–264)	122*,** (32–152)	197 (17–340)	130*,** (32–182)	251*,** (12–360)	149*,** (75–235)	237*,** (32–240)	173* (87–485)
200	133*,** (25–220)	62*,** (20–122)	164* (5–292)	122*,** (42–157)	198*,** (10–242)	129*,** (50–177)	183*,** (5–256)	142*,** (75–242)
400	91*,** (7–137)	53*,** (20–75)	107*,** (17–215)	102*,** (45–165)	159*,** (10–292)	107*,** (42–167)	151*,** (7–280)	118*,** (32–200)

* $P < 0.05$ (compared to basic culture where 0.25 U/ml EPO was added without change in TNFα concentration)
** $P < 0.05$ (compared to basic culture where no TNFα was added and no change in EPO concentration)

r-h-EPO, and 50 U/ml and 400 U/ml TNFα gave suppression of 28% and 49%, respectively, compared to the culture without TNFα. Increasing the concentration of r-h-EPO from 0.25 U/ml to 2 U/ml resulted in complete correction of inhibition when 50 U/ml TNFα was used and partial correction of the TNFα suppression to 28% when 400 U/ml TNFα was added to the cultures. Similar results were obtained in group 2.

The correction of TNFα inhibition also occurred in BFUe cultures. Regarding BFUe (in group 1), incubation with 0.25 U/ml r-h-EPO, and 50 U/ml and 400 U/ml TNFα resulted in suppression of 33% and 61%, respectively, as compared to the cultures without TNFα. Increasing the concentration of r-h-EPO from 0.25 U/ml to 2 U/ml diminished the suppression to 17% (50 U/ml TNFα) and 55% (400 U/ml TNFα). BFUe growth was significantly more inhibited as compared to CFUe at similar TNFα concentrations. CFUe suppression was counteracted more by higher concentrations of r-h-EPO than was BFUe suppression.

Discussion

Many theories exist to explain the pathogenesis of ACD, including an impaired EPO production, decreased bone marrow sensitivity to EPO and inhibition of erythroid progenitors by cytokines such as TNFα. Our study demonstrated that TNFα indeed suppressed erythropoiesis (CFUe and BFUe) in vitro and this inhibition was partly reversed by increased concentrations of r-h-EPO.

The mechanism by which this effect occurs is unknown at the present. It could be speculated that EPO causes a down-regulation of TNFα receptor expression on BFUe and CFUe, thus reducing the response to the inhibitor. Alternatively, TNFα may down-regulate EPO receptor expression on BFUe and CFUe, with the CFUe and BFUe requiring higher r-h-EPO concentrations for optimum colony formation. Possible interactions of EPO and TNFα, as well as other cytokines such as IFNγ [27], on receptor levels will be subjects of future studies. On the other hand, the inhibitory effects of TNFα could be enhanced by a TNFα-mediated release of other cytokines in the cultures, such as IL-1 and IFNγ, causing additional suppression of erythropoiesis [28].

We demonstrated that the CFUe colony growth inhibition by a low concentration of TNFα (50 U/ml) was completely restored when r-h-EPO was increased from 0.25 U/ml to 2 U/ml. Apparently, in cultures where higher concentrations of TNFα were added, r-h-EPO was not able to counteract inhibition completely. Similar results have been shown by Means [27] who showed that r-h-IFNγ-related inhibition of CFUe colony growth is reversed by increased concentrations of r-h-EPO. The late erythroid progenitors (CFUe) are more dependent on EPO than earlier progenitors (BFUe), which also require other growth factors (such as IL-3) for differentiation and proliferation. This may explain the better correction of TNFα-inhibited CFUe growth with the addition of r-h-EPO as compared to BFUe growth in our study. We did not establish any differences in CFUe and BFUe growth between RA patients with ACD and RA patients without anaemia. In this in vitro culture system we used much higher concentrations of TNFα and EPO than can be expected to be present in vivo, which might explain why no differences were observed between the two groups.

Johnson et al. [25] have reported that, in TNFα-treated mice, EPO abrogates the TNFα suppressive effect in a dose-dependent manner. This observation has been sustained by observations [24] that EPO treatment prevents a decrease in the haematocrit during chronic administration of TNFα [24]. In RA patients, circulating levels of TNF are much lower (max. 50 pg/ml) than those used in the above mentioned study [25] but the local concentra-

tion of TNFα in the bone marrow might be higher and thus participate in the pathogenesis of ACD in RA.

Our in vitro data supported the hypothesis of lower levels of EPO [18], as well as elevated circulating TNFα serum levels [14], playing an important role in the development of ACD in patients with RA. Further efforts should be directed to demonstrating the relationship between in vitro results and clinical observations.

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References

1. Dayer JM, Beutler B, Cerami A (1985) Cachectin/tumor necrosis factor stimulates collagenase and prostastaglandin E_2 production by human synovial cells and dermal fibroblasts. J Exp Med 162:2163–2168
2. Saklatvala J (1986) Tumor necrosis factor alpha stimulates resorption and inhibits synthesis of proteoglycan in cartilage. Nature 322:547–549
3. Buchan G, Barret K, Turner M, Chantry D, Maini RN, Feldmann M (1988) Interleukin-1 and tumor necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha. Clin Exp Immunol 73:449–455
4. Ridderstad A, Abedi-Valugerdi M, Moller E (1991) Cytokines in rheumatoid arthritis. Ann Med 23:219–223
5. Gordon LI, Miller WJ, Branda RF, Zanjani ED, Jacob HS (1988) Regulation of erythroid formation by bone marrow macrophages. Blood 55:1047–1050
6. Feldman M (1989) Molecular mechanisms involved in human autoimmune disease: relevance of chronic antigen presentation, class II expression and cytokine production. Immunology 2:66–71
7. Akahane K, Hosoi T, Urabe A, Kawakami M, Takaku F (1987) Effects of recombinant tumor necrosis factor (rhTNF) on normal human and mouse haematopoietic progenitor cells. Int J Cell Cloning 5:16–26
8. Roodman GD, Bird A, Hutzler D, Montgomery W (1987) Tumor necrosis factor alpha and haematopoietic progenitors: effects of tumor necrosis factor alpha on the growth of erythroid progenitors CFU-E and BFU-E and the haematopoietic cell lines K562, HL60, and HEL cells. Exp Hematol 15:928–935
9. Broxmeyer HE, Williams DE, Lu L, Cooper S, Anderson SL, Beyer GS, Hoffman R, Rubin BY (1986) The suppressive influences of human tumor necrosis factor on bone marrow haematopoietic progenitor cells from normal donors and patients with leukaemia: synergism of tumor necrosis factor and interferon-gamma. J Immunol 136:4487–4495
10. Means RT, Dessypris EN, Krantz SB (1990) Inhibition of human erythroid colony-forming units by tumor necrosis factor accessory cells. J Clin Invest 86:538–541
11. Means RT, Krantz SB (1993) Inhibition of human erythroid colony-forming units by tumor necrosis factor alpha requires beta interferon. J Clin Invest 91:416–419
12. Backx B, Broeders L, Bot FJ, Löwenberg B (1991) Positive and negative effects of tumor necrosis factor on colony growth from highly purified normal marrow progenitors. Leukemia 5:66–70
13. Johnson RA, Waddelow TA, Caro J, Roodman GD (1989) Chronic exposure to tumor necrosis factor alpha in vivo preferentially inhibits erythropoiesis in nude mice. Blood 74:130–138
14. Vreugdenhil G, Löwenberg B, Van Eijk HG, Swaak AJG (1992) Tumor necrosis factor alpha is associated with disease activity and the degree of anaemia in patients with rheumatoid arthritis. Eur J Clin Invest 22:488–493
15. Maury CJP, Lähdevitra J (1990) Correlation of serum cytokine levels with haematological abnormalities in human immunodeficiency virus infection. J Int Med 227:253–257
16. Boyd HK, Lappin TRJ, Bell AL (1991) Evidence for impaired erythropoietin response to anaemia in rheumatoid arthritis. Br J Rheumatol 30:255–259
17. Remacha AF, Rodríguez-de la Serna A, Gracía-Dié F, Geli C, Díaz C, Gimferrer E (1992) Erythroid abnormalities in rheumatoid arthritis: the role of erythropoietin. J Rheumatol 19:1687–1691
18. Jelkman W, Pagel H, Wolff M, Fandrey J (1991) Monokines inhibit erythropoietin production in human hepatoma cultures and isolated perfused rat kidneys. Life Sci 50:301–308
19. Vreugdenhil G, Wognum AW, Van Eijk HG, Swaak AJG (1990) Anaemia in rheumatoid arthritis: the role of iron, vitamin B_{12} and folic acid deficiency and erythropoietin responsiveness. Ann Rheum Dis 49:93–98
20. Salvarani C, Casali B, Salvo D, Brunati C, Macchioni PL, Massai G, Lasagni D, Rivasi P, Portoli I (1991) The role of interleukin 1, erythropoietin and red cell bound immunoglobulins in the anaemia of rheumatoid arthritis. Clin Exp Rheumatol 9:241–246
21. Means RT, Olsen NJ, Krantz SB, Dessypris EN, Graber SE, Stone WJ, O'Neil VL, Pincus T (1989) Treatment of the anaemia of rheumatoid arthritis with recombinant human erythropoietin: clinical and in vitro studies. Arthritis Rheum 32:638–642
22. Pincus T, Olsen NJ, Russell IJ, Wolfe F, Harris ER, Schnitzer TJ, Boccagno JA, Krantz SB (1990) Multicenter study of recombinant human erythropoietin in correction of anemia in rheumatoid arthritis. Am J Med 89:161–168
23. Birgegard G, Gudbjronsson B, Hallgren R, Wilde L (1991) Anaemia of chronic inflammatory arthritides: treatment with recombinant human erythropoietin. Contrib Nephrol 88:295–303
24. Vreugdenhil G, Manger B, Nieuwenhuizen C, Feelders RA, Van Eijk HG, Swaak AJG (1992) Iron stores and serum transferrin receptor levels during recombinant human erythropoietin treatment of anaemia in rheumatoid arthritis. Ann Hematol 65:265–268
25. Johnson CS, Cook CA, Furmanski P (1990) In vivo suppression of erythropoiesis by tumor necrosis factor-α (TNFα): reversal with exogenous erythropoietin (EPO). Exp Hematol 18:109–113
26. Reference deleted
27. Means RT, Krantz SB (1991) Inhibition of human erythroid colony-forming units by γ interferon can be corrected by recombinant human erythropoietin. Blood 78:2564–2567
28. Furmanski P, Johnson CS (1990) Macrophage control of normal and leukemic erythropoiesis: identification of the macrophage-derived erythroid suppressing activity as Interleukin-1 and the mediator of its in vivo action as tumor necrosis factor. Blood 75:2328–2334