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Anaemia of chronic disease in rheumatoid arthritis

Raised serum interleukin-6 (IL-6) levels and effects of IL-6 and anti-IL-6 on in vitro erythropoiesis

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Summary. Serum and bone marrow from 21 patients with rheumatoid arthritis (RA) were studied in order to establish the pathogenetic role of interleukin-6 (IL-6) in anemia of chronic disease (ACD). Erythroid colony growth, using burst forming units of erythroblasts (BFUe) as a parameter, was impaired in ACD and not in nonanemic RA controls. Serum IL-6 was elevated in ACD and it correlated well with parameters of disease activity such as erythrocyte sedimentation rate and C-reactive protein. IL-6 addition to bone marrow cultures had inconsistent effects while anti-IL-6 addition resulted in impaired erythroid colony growth, suggesting stimulatory effects of IL-6 produced in the medium, which may be masked by simultaneous production of cytokines with suppressive effects. It was concluded that elevated serum IL-6 in ACD reflects disease activity. It probably plays no pathogenetic role in ACD. Its stimulatory effects on erythroid growth might counteract suppressive effects of other interleukins.

Key words: Rheumatoid arthritis – Anemia – Erythroid colony growth – Interleukin-6, IL-6, anti-IL-6

Introduction

Anemia is frequently seen in patients with active rheumatoid arthritis (RA) [1]. Many causes of anemia are associated with RA, such as deficiencies of iron [2], vitamin B12 [3], and folic acid [4]. The most frequent type of anemia seen in RA, however, is the anemia of chronic disease (ACD) [5]. Many studies have been carried out to examine pathogenesis of ACD in RA. Decreased iron release from the mononuclear phagocyte system (MPS) [6, 7], decreased iron absorption [8], and decreased erythropoietin responsiveness to the anemia [9] have been claimed to play a role in the pathogenesis of ACD. More recently, investigations have also been concerned with a possible role of interleukins as mediators. For instance, interleukin-1 (IL-1) and tumor necrosis factor (TNF) were able to suppress erythropoiesis in vitro [10, 11].

Interleukin-6 (IL-6) is a monokine with biological activities related to inflammatory responses [12, 13]. IL-6 levels were elevated in serum and synovial fluid of patients with active RA [14, 15]. About the effects of IL-6 on bone marrow only few data exist [16].

Here we report experiments dealing with a possible role of IL-6 in determining anemia in RA.

Patients and methods

Serum from 21 patients (6 male, 15 female) with classical or definite RA [17] and bone marrow from 21 RA patients and 5 normal donors were studied after obtaining patients' written informed consent. RA patients were divided into two groups: group I consisted of 9 nonanemic patients and group II of 12 patients with ACD. Patients who had iron, vitamin B12, or folic acid treatment recently, or patients with a present or past ulcer history, hematuria, hypermenorrhea, positive occult fecal blood test, hemolysis, iron, vitamin B12, or folic acid deficiency, or decreased creatinine clearance were excluded. Patients using corticosteroids or cytostatic drugs were also excluded. Mean overall disease duration had been 7 years (range 4–17); 74% used long-acting antirheumatic drugs and 88% used nonsteroidal antiinflammatory drugs. Mean age was 64 years. These characteristics did not differ between groups I and II.

Laboratory procedures. Hemoglobin (Hb), hematocrit (Ht), reticulocytes, and ferritin and erythrocyte sedimentation rates (ESR) were measured using standard laboratory procedures. C-reactive protein (CRP) was assessed using immunodiffusion techniques and Rose test using sensitized sheep erythrocytes. A titer over 1/32 was considered positive [18].

IL-6 in serum was measured in the B9 assay [19]. Serum was heated for 30 min at 56 °C and a titration was added to 5000 B9 cells and compared with standard IL-6 preparation. After 3 days proliferation was measured by thymidine incorporation; 1 U/ml is a concentration that leads to one-half maximal proliferation. In 100 healthy individuals serum levels were less than 10 U/ml.

Bonel marrow was aspirated after posterior superior iliac crest puncture. Iron content was measured using Perls' Prussian blue

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Table 1. Erythrocyte parameters, parameters of disease activity, IL-6, and burst forming units (BFUe) per 10^5 cells in RA patients without anaemia and ACD

	Hb (mmol/)	Ht (L/L)	Retics (0/00)	BFUe (col. per 10^5 cells)	ESR (mm/h)	CRP (mg/l)	Rose reciprocal titer	IL-6	Ferritin (μg/1)
Nonanemic RA ($n=9$)	7.9 (7.5–8.3)	0.39 (0.35–0.42)	17 (5–39)	260 (215–391)	34 (21–60)	8 (4–45)	32 (0–256)	0 (0–11)	39 (12–86)
ACD RA	6.6*	0.31*	17	191**	74*	62**	32	29***	108**
($n=12$)	(5.4–7.3)	(0.28–0.34)	(1–28)	(123–108)	(40–105)	(10–121)	(0–256)	(0–82)	(39–410)

* $P<0.001$, ** $P<0.01$, *** $P<0.10$. Values are expressed as median with range

Table 2. Standard BFUe count (no addition; counts per 10^5 cells incubated) and absolute (abs) and relative (%) change in BFUe count after addition of IL-6 (1000 U/ml) and anti-IL-6 (1000 (U/ml) to the cultures in controls, nonanemic, and anemic RA patients

Addition	Controls ($n=5$)		Nonanemic RA ($n=3$)		ACD RA ($n=8$)	
	abs	%	abs	%	abs	%
None	356 (254–396)	100	246 (242–282)	100	173 (12–440)	100
IL-6	266* (225–266)	80±20* (57–105)	294 (168–318)	111±2* (109–113)	155 (6–471)	91±21 (83–107)
Anti-IL-6	219** (168–361)	72±16*** (55–92)	228* (180–244)	86±10* (74–99)	135* (8–329)	72±13*** (43–83)

* $P<0.10$, ** $P<0.05$, *** $P<0.01$; difference compared with standard BFUe count. Values expressed as median with range (percentages as mean and standard deviation

staining. A stainable iron content of 0–1 was considered to signify iron deficiency [20].

A cell suspension was prepared from 20 ml bone marrow collected in Hanks' balanced salt solution (HBSS) with heparin diluted in HBSS and layered over a Ficoll gradient (1.077 g/cm^2, Nycomed, Oslo, Norway). After centrifugation the mononuclear cells were harvested, washed twice in HBSS, and resuspended in HBSS [21]. A cell suspension of 10^5 cells was added to a mixture of Iscove's mediated Dulbecco medium (IMDM; 0.4 ml) 2% methylcellulose, 0.3 ml fetal calf serum, 0.1 ml mixture (containing BSA, transferrin lecithin, sodium selenite, and 2-mercaptoethanol), and 0.015 ml erythropoietin 1 U/L). This volume was divided over four Petri dishes which were incubated at 37°C and 100% humidity in an environment of 5% CO_2 in air. Burst forming units of erythroblasts (BFUe; containing 50 or more cells) were counted after 14 days.

In three patients from group I, eight from group II, and in five normal donors IL-6 (1000 U/ml) and anti-IL-6 (1000 U/ml; synthesized by using a recombinant DNA technique; Central Laboratory of the Netherlands Red Cross and Blood Transfusion Service, Amsterdam, The Netherlands) were added to the BFUe cultures to evaluate their effects on in vitro erythropoiesis. In seven patients (two in group I; five in group II) twice the amount of IL-6 or anti-IL-6 was added.

Statistics. Student's *t*-test was used for normally distributed data and the Mann-Whitney U-test was used for nonparametrical data. Data were correlated using Spearman's coefficient of correlation. A *P*-value of less than 0.05 was considered significant.

This protocol was accepted by the Medical Ethics Committee of the Department of Rheumatology of the Dr. Daniel den Hoed Clinic.

Results

Parameters of erythropoiesis, disease activity and IL-6 in RA patients without and with anemia

The BFUe count was significantly lower in group II compared with group I (Table 1). Reticulocytes did not differ between the two groups.

The ESR and CRP were significantly higher in group II. Hb correlated negatively with ESR ($r=-0.71$; $P<0.001$) and CRP ($r=-0.68$; $P<0.005$). The BFUe count correlated negatively with ESR ($r=-0.65$; $P>0.005$) and CRP ($r=-0.66$; $P<0.01$). No differences in Rose titer were found.

Serum IL-6 tended to be higher in group II. IL-6 correlated positively with ESR ($r=0.46$; $P<0.025$) and CRP ($r=0.52$; $P<0.025$) and negatively with Hb ($r=-0.41$; $P<0.05$) but not with BFUe.

Effects of addition of IL-6 and anti-IL-6 to bone marrow cultures (Table 2)

The BFUe count was higher in controls compared with ACD patients ($P<0.05$). The difference between controls (healthy donors) and nonanemics was not significant.

IL-6 addition slightly inhibited BFUe count in controls but stimulated growth in nonanemics. In seven cultures twice the amount of IL-6 was added (two controls, five ACD; not shown). Mean percentual BFUe count was 109% ± 18% compared with control BFUe count.

Anti-IL-6 addition inhibited BFUe count in all groups, most pronounced in controls and ACD. Doubling the amount of anti-IL-6 added had no further inhibitory effect on BFUe count (mean 82 ± 16%; $P < 0.01$; not shown).

Discussion

We evaluated whether in vitro erythroid colony growth was reduced in patients with RA and ACD and examined a potential pathogenetic role of IL-6 in ACD.

Erythroid colony growth, measured by BFUe count, was impaired in patients with RA and ACD and not in nonanemic RA patients. Reticulocyte count was the same in nonanemic and anemic RA patients, which further points to an inadequate bone marrow response in the anemia. A inverse correlation was found between parameters of RA activity, i.e., ESR and CRP and BFUe count and Hb. It therefore appears that RA activity determines the degree of anemia in ACD by inhibition of erythroid growth at stem cell level. Others have also described an inverse relationship between Hb and RA disease activity [9]. Defective erythropoietin response to the anemia is claimed to contribute to the pathogenesis of ACD [9], while others point to suppressive effects of interleukins such as interleukin-1 and tumor necrosis factor alpha (TNFα) on erythroid colony growth [10, 11].

IL-6 is a monokine with biological activities related to inflammatory responses [12, 13]. It was shown that IL-6 levels were evaluated in serum and synovial fluid of patients with active RA [14, 15]. In another study among patients with systemic lupus erythematosus it was also found that IL-6 correlated with CRP, although in these patients, in contrast to RA patients with active RA, serum levels were low [22]. It was suggested that IL-6 determined CRP response.

We found that serum IL-6 levels correlated positively with parameters of RA activity, i.e., ESR and CRP, which confirms its role in the inflammatory response in RA. Serum IL-6 correlated negatively with Hb and it was elevated in most of our ACD patients. Since we found an inverse relationship between RA disease activity and BFUe count, it was assumed that IL-6 might be a mediator of impaired erythroid colony growth.

IL-6 and anti-IL-6 antibodies were added to the bone marrow cultures in order to establish their effects on erythroid colony growth. IL-6 addition resulted in a slight inhibition of BFUe growth in controls and ACD. In nonanemic controls in contrast it stimulated erythroid growth. After addition of anti-IL-6 antibody erythroid colony growth was impaired in all cases. Addition of twice the amount of IL-6 or anti-IL-6 did not increase the in vitro effects in either case. From these data it is concluded that anti-IL-6 might have inhibitory effects of erythroid colony growth. Probably IL-6 is produced by bone marrow cells. Possibly the stimulatory effects of locally produced IL-6 are neutralized by the addition of anti-IL-6 while IL-6 addition in the amount used here has no further effects on erythroid growth. The anti-IL-6 antibody specificity is not entirely known. So although a consistent inhibition or erythropoiesis occurred it is not known whether antibodies with a similar isotype as anti-IL-6 might have caused the same suppression. If so it could also be that, for instance, effects of other cytokines are inhibited. IL-6 production probably is enhanced by IL-1, and TNF [23–25]. It is therefore not unlikely that IL-6 production is stimulated by IL-1 and TNF production in the marrow cultures. These cytokines inhibit erythropoiesis [10, 11] masking potential stimulative effects of IL-6. It might even be speculated that IL-6 induces production of other cytokines with suppressive effects on erythroid growth. This might explain the inconsistent effects of IL-6 addition in the different groups. IL-6 has been found to trigger primitive hematopoietic cells into cell cycle in vitro, most pronounced in the presence of IL-3 [16], whereas others found that IL-6 had no effects on erythroid growth in acute myeloblastic leukemia [26]. It is therefore unlikely that IL-6 – shown to be raised in serum of RA patients with ACD – is a cause of ACD in RA. In contrast, it might be speculated, based on the findings of anti-IL-6 addition, that IL-6 might counteract suppressive effects of other interleukins such as IL-1 [10] and TNFα [11], which enhance IL-6 production [24–26], on erythroid colony growth.

In conclusion, these preliminary data show that erythroid colony growth is impaired in ACD. Serum IL-6 correlated with RA activity and was elevated in ACD but it had no pathogenetic role in ACD since in vitro its antibody rather had suppressive than stimulatory effects on erythroid growth while the effects of IL-6 addition were inconsistent.

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References

1. Mowat AG (1971) Hematologic abnormalities in rheumatoid arthritis. Semin Arthritis Rheum 1:383–390
2. Hansen TM, Hansen NE, Birgens HS, Hølund B, Lorenzen I (1983) Serum ferritin and the assessment of iron deficiency in rheumatoid arthritis. Scand J Rheumatol 12:353–359
3. Couchman KG, Bieder L, Wigley RD, Glenday AG (1968) Vitamin B12 absorption and gastric antibodies in rheumatoid arthritis. NZ Med J 68:153–156
4. Urban DJ, Ibbottson RN, Horwood J, Milazzo S, Robson HN (1966) Folic acid deficiency in rheumatoid arthritis: relation of levels of serum folic acid to treatment with phenyl-butazon. Br Med J 1:765–768
5. Cartwright GE, Lee GR (1971) The anemia of chronic disorders. Br J Haematol 21:147–152
6. Beamish MR, Davis AG, Eakins JD, Jacobs A (1971) The measurement of reticuloendothelial iron release using iron dextran. Br J Haematol 21:617–622
7. Bentley DP, Cavill I, Rickets C, Peake S (1979) A method for the investigation of reticuloendothelial iron kinetics in man. Br J Haematol 43:619–624

8. Benn HP, Drews J, Randzio G, Jenssen JM, Löffler H (1988) Does active rheumatoid arthritis affect intestinal iron absorption? Ann Rheum Dis 47:144–149
9. Birgegard G, Hällgren R, Caro J (1987) Serum erythropoietin in rheumatoid arthritis and other inflammatory arthritides: relationship to anemia and the effect of antiinflammatory treatment. Br J Haematol 65:479–483
10. Maury CPJ, Andersson LC, Teppo AM, Partanen S, Juvonen E (1988) Mechanisms of anaemia in rheumatoid arthritis: demonstration of raised interleukin-1 β concentrations in anaemic patients and of interleukin-1 mediated suppression of normal erythropoiesis and proliferation of human erythroleukemia (HEL) cells in vitro. Am Rheum Dis 47:972–987
11. Roodman GD (1987) Mechanisms of erythroid suppression in anaemia of chronic disease. Blood Cells 13:171–184
12. Geiger T, Anders T, Klapproth J, Hirano T, Kishimoto T, Heinrich PL (1988) Induction of rat acute-phase proteins by interleukin-6 in vivo. Eur J Immunol 18:717–721
13. Nijsten MWN, Groot ER de, Duis HJ ten, Klasen HJ, Hack CE, Aarden LA (1987) Serum levels of interleukin-6 and acute phase response. Lancet II:921
14. Swaak AJG; Van Rooyen A, Nieuwenhuis E, Aarden LA (1988) Interleukin-6 (IL-6) in synovial fluid and serum of patients with rheumatic diseases. Scand J Rheumatol 17:469–474
15. Houssian FA, De Vogelaer JP, Van Damme J, De Deuxchaisnes CN, Van Snick J (1988) Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. Arthritis Rheum 31:784–788
16. Leary AG, Ikebuchi K, Hirui Y, Wong GG, Yang YC, Clark SC, Ogawa M (1988) Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human haematopoietic stem cells: comparison with interleukin-1 alpha. Blood 71:1759–1763
17. Ropes MW (1959) Diagnostic criteria fro rheumatoid arthritis. Ann Rheum Dis 18:49–51
18. Rose HM, Ragan C, Pearce E, Lepman MV (1949) Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis. Proc Soc Exp Biol Med 68:1–12
19. Aarden LA, Groot ER de, Schaap OL, Landsdorp PM (1987) Production of hybridoma growth factor by human monocytes. Eur J Immunol 17:1411–1416
20. Lundin P, Persson E, Weinfeld A (1964) Comparison of hemosiderin estimation in bone marrow sections and bone marrow smears. Acta Med Scand 175:383–390
21. Bot FJ, Dorssers L, Wagemaker G, Löwenberg B (1988) Stimulating spectrum of human recombinant multi-CSF (IL-3) on human marrow precursors: importance of accessory cells. Blood 71:1609–1614
22. Swaak AJG, Van Rooyen A, Aarden LA (1989) Interleukin-6 (IL-6) and acute phase proteins in the disease course of patients with systemic lupus erythematosus. Rheumtol Int 8:263–268
23. Content J, Wit L de, Powpart P, Opdenakker G, Damme J van, Billiau A (1985) Induction of a 26 kDa protein mRNA in human cells treated with an interleukin-1 related leukocyte derived factor. Eur J Biochem 152:253–257
24. Helle M, Brakenhoff JPJ, Groot ER de, Aarden LA (1988) Interleukin-6 is involved in interleukin-1 induced activities. Eur J Immunol 18:957–959
25. Haegeman G, Content J, Volckaert G, Derynck R, Tavernier J, Fiers W (1986) Structural analysis of the sequence coding for an inducible 26 kDa protein in human fibroblasts. Eur J Biochem 159:625–632
26. Hoang T, Homan A, Goncalues O, Wong GG, Clark SC (1988) Interleukin-6 enhances growth factor dependant proliferation of the blast cells of acute myelocytic leukemia. Blood 72:823–826