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IL-6-induced anaemia in rats: possible pathogenetic implications for anaemia observed in chronic inflammations

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SUMMARY

Anaemia of chronic disease (ACD) is frequently found in rheumatoid arthritis (RA). In the pathogenesis of ACD both cytokines, such as tumour necrosis factor-alpha (TNF-α), IL-1 and Il-6 as well as a relative deficiency of erythroprotein (EPO), are thought to play a key role. In the present study the role of IL-6 in the pathogenesis of this anaemia was investigated. IL-6 was administered intraperitoneally to rats for 14 sequential days. It appeared that IL-6 was able to induce anaemia. No evidence for suppression of bone marrow erythropoiesis or enhanced sequestration of erythrocytes in the liver was found. However, decreased plasma and bone marrow iron contents were observed in anaemic rats. Blood loss in intestinal tissue was demonstrated using erythrocyte labelling with ^{99m}technetium. Histologically this was associated with inflammatory cell infiltration, oedema and bleeding in the intestinal wall. In conclusion, IL-6 induced anaemia in rats. This anaemia was caused by intestinal blood loss.

Keywords IL-6 anaemia chronic disease anaemia of chronic disease

INTRODUCTION

Anaemia of chronic disease (ACD) frequently accompanies chronic inflammatory, neoplastic and infectious disorders [1]. Anaemia in rheumatoid arthritis (RA) often serves as a model for ACD, since 60–70% of anaemic RA patients can be classified as ACD [2,3]. The pathogenesis of ACD in RA is multifactorial. Proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), IL-1 and IL-6 are supposed to participate in the development of anaemia by modulation of iron metabolism [4,5] and suppression of bone marrow erythropoiesis [6–8]. These cytokines were also shown to suppress, both *in vitro* and *in vivo*, erythropoietin (EPO) production [9].

IL-6 is a potent cytokine with a key role in the formation of inflammatory responses [10], namely by stimulating the synthesis of the acute-phase proteins such as C-reactive protein (CRP). Elevated levels of CRP are found in ACD patients compared with non-anaemic RA patients which correlated well with IL-6 serum levels and disease activity [11].

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Administration of IL-6 induced anaemia in experimental animals [12–14] and in humans [15,16]. The pathogenesis of this IL-6-induced anaemia has not been fully elucidated. It has been suggested that modulation of erythropoiesis, sequestration of erythrocytes in the reticuloendothelial system, blood loss and/or haemodilution, secondary to a shift of fluid to the intravascular space, may be responsible for this anaemia [15–17].

It remains uncertain how these observations could contribute to explain the role of IL-6 in pathogenesis of ACD in RA. In RA high serum concentrations of IL-6 are generally found [11,18,19], and the administration of anti-IL-6 MoAbs in patients with a severe RA caused a significant increase of haemoglobin (Hb) in anaemic patients [20]. Furthermore, a transient beneficial effect on anaemia was reported in a patient with Castleman's disease (angiofollicular lymphoid hyperplasia) treated with anti-IL-6 [21].

Therefore, the aim of the present study was to investigate the effects of IL-6 on the haematological parameters in an experimental animal model, and to study the nature of IL-6-induced anaemia. Rats were treated with IL-6 in order to study the effects of peripheral blood parameters and on bone marrow. Furthermore, an additional erythrocyte-labelling study was performed in IL-6-treated rats to investigate the possibility of erythrocyte sequestration.

MATERIALS AND METHODS

Experimental animals

The experiments were performed in 12–14-week-old female rats (body weight 120–150 g) of the inbred Brown Norway rat strain (BN/Rij). The animals were bred under specific pathogen-free conditions in the breeding colony of 'Harlan' (Rijswijk, The Netherlands) and maintained under clean conventional conditions.

Cytokines

Recombinant human (rh) IL-6 (glycosylated, recombinant human IL-6; Sigosix; Ares-Serono, Geneva, Switzerland) with a specific activity of $23{\cdot}3 \times 10^6$ U/mg, purity >99% and a not-detectable endotoxin content (<0·125 endotoxin units/mg) was used. The specific activity of rhEPO (Boehringer, Mannheim, Germany) was 10^5 U/mg. The recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) (10^7 U/mg) was obtained from Behring Werke AG (Marburg, Germany).

Treatment

Experimental design I. Two groups of six rats each were treated for 14 days and monitored for an additional 11 days. One group (IL-6 group) was injected intraperitoneally with IL-6 (100 μg/kg) twice daily, and control rats (control group) were injected daily intraperitoneally with 0·9% NaCl.

Experimental design II. Two additional groups of eight rats were used to perform an erythrocyte labelling study. The first group received one dose of IL-6 (200 μg/kg), whereas the second group served as controls and received 0·9% NaCl intraperitoneally 1 h before erythrocyte labelling. Twelve hours later four rats of the IL-6-treated group received a second and 24 h later a third dose of IL-6 (200 μg/kg).

Assessments

Blood analysis. At the baseline and at days 1, 4, 7, 14, 18 and 25 peripheral blood samples for complete blood counts (Hb, haematocrit (Ht), erythrocytes, leucocytes, platelets and plasma iron) were obtained by tail vein clipping and analysed using a haemocytometer (Sysmex F-800). Reticulocyte percentages were determined by standard counting after staining with thiazole orange and measured with FACScan and the Retic-count computer software. Heparinized plasma was obtained by centrifugation and kept frozen at −20°C. A sensitive ELISA, developed for human IL-6 as described previously [22], was used to determine IL-6 plasma concentration.

Bone marrow analysis. After 14 days of treatment, bone marrow cells of three animals of each group were obtained by flushing the femurs with α MEM (GIBCO, Gaithersburg, MD), pooled and cultured in triplicate 35-mm tissue culture dishes in 0·8% methylcellulose, 20% horse serum, 10^{-6} M β-mercaptoethanol, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were supplemented with rmGM-CSF (500 U/ml) for granulocyte-macrophage colony formation (CFU-GM). Colonies were counted after 8 days. For erythroid burst-forming units (BFU-E) and colony forming units (CFU-E) cells were cultured as described previously [23]. After 3 days culturing, under 7% CO_2 in air at 37°C and 100% humidity, cell groups of five cells or more exhibiting a red colour were counted as colonies derived from CFU-E; after culture for 11 days colonies derived from BFU-E were enumerated.

The iron contents of the same pooled bone marrows were measured by staining of the bone marrow smears with Perl's Prussian blue in triplicates. A semiquantitative scale (0–4) was used; 0 = no stainable iron, 1 = minimal amount, 2 = slight small and patchy content, and 3–4 = normal to increased stainable iron.

In vivo *labelling of erythrocytes with* ^{99m}*technetium*

Amerscan (Amersham International plc, Aylesbury, UK) in a calculated dose of 0·3 ml/kg was used for *in vivo* loading of erythrocytes with stannous ions, prior to ^{99m}technitium (^{99m}Tc) labelling. Between 20 and 40 min following i.v. administration of Amerscan, ^{99m}Tc (22·5 MBq/rat) was injected intravenously. A few minutes after injection (time point 0) and 2, 4 and 6 h post-injection, radioactivity in the heart and liver was measured using a gamma-camera. The levels of radioactivity measured in the heart correspond with the number of the erythrocytes in the circulation, whereas the liver is associated with the amount of erythrocyte sequestration. No measurements could be performed in the spleen because of the overlap of that organ with the left kidney. This procedure was performed in the control group and IL-6 treated rats, after one as well as after three doses of IL-6.

Histologic assessments

Following ^{99m}Tc erythrocyte imaging (24 h after administration of IL-6) liver, spleen, lung and intestinal tissue specimens of IL-6 treated and control animals were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained with haematoxylin–eosin, and histologically analysed. All intestinal specimens were evaluated for inflammatory cell infiltration, ulceration, oedema and bleeding. Histological scoring ranged form 0 to 3 (−, normal; +, mild; ++, moderate; +++, severe).

Statistical analysis

Student's *t*-test or Wilcoxon rank sum test in case of quantitative variables and Fischer's exact test in case of qualitative variables was used to assess the level of significance between treatment groups.

RESULTS

IL-6 plasma levels

At baseline no IL-6 was measured in plasma samples. Only the IL-6 group showed an increase in IL-6 during treatment. At day 1 of treatment a median IL-6 level of 1048 (532–9309) pg/ml was found in the IL-6 group, and similar levels were observed during the treatment period.

Effects on peripheral blood

IL-6 induced anaemia in animals of the IL-6 group. On days 1, 4, 7 and 14 Hb of IL-6 group differed significantly from the control group (Fig. 1a). Similar results were obtained for Ht and erythrocytes (data not shown).

Administration of IL-6 resulted in an increase of the reticulocyte number and platelets compared with control animals (Fig. 1b,c).

On day 14 of treatment the number of leucocytes increased by 68% in the IL-6 groups compared with the baseline. The difference between groups was statistically significant ($P < 0{\cdot}01$).

Significant ($P < 0{\cdot}01$) decrease in plasma iron concentration was observed in the IL-6 group compared with the control group (Fig. 1d). Plasma iron concentration remained lower until the end

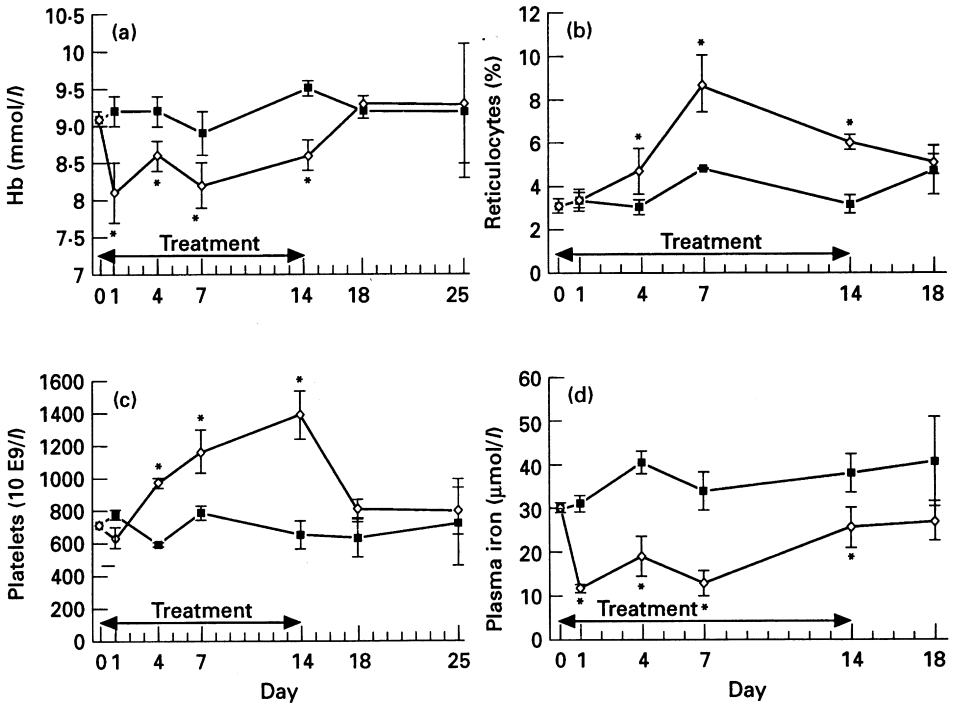

Fig. 1. Time courses for the effect of IL-6 on different haematological and serological parameters: (a) haemoglobin (Hb); (b) reticulocytes; (c) platelets; (d) plasma iron. Experimental animals were given twice daily either an i.p. injection of IL-6 (100 μg/kg) or 0·9% NaCl, on day 0–14. The points present mean ± s.e.m. for six animals assayed individually at each time. *Statistically significant ($P < 0{\cdot}01$) difference between groups. ◇, IL-6; ■, controls.

of treatment (day 14) and returned to baseline values later in the follow up (day 18).

Effect on bone marrow

After 14 days of treatment, a higher number of CFU-GM was observed in the IL-6 group ($P < 0{\cdot}001$ compared with control animals) (Fig. 2). Stimulation of BFU-E growth was found in the IL-6 group compared with the control group ($P < 0{\cdot}05$). The groups were comparable regarding the number of CFU-E.

Furthermore, administration of IL-6 diminished the amount of stainable bone marrow iron (semiquantitative score 2) compared with the control group (score 3).

Effect on ^{99m}Tc erythrocyte distribution

A significant decrease of 12% ($P < 0{\cdot}001$ compared with controls) in the level of radioactivity (ct/min), as measured in the heart, was observed in IL-6-treated animals, indicating a decreased number of erythrocytes in the circulation. The difference appeared as early as time point 0 (1 h after administration of the first dose of IL-6 intraperitoneally) and remained at the same level at consecutive time points (2, 4 and 6 h). Animals with different doses of IL-6 did not show any differences in the level of radioactivity measured in the heart (Fig. 3). Administration of IL-6 resulted in diminished radioactivity in the liver, compared with controls, making sequestration of ^{99m}Tc erythrocytes in the liver unlikely (Fig. 3). Furthermore, there was evidence of accumulation of radioactivity in abdominal organs, suggestive for local blood loss (Fig. 4).

Histological assessments

No pathologic changes were observed in the liver, spleen or lung tissue of rats treated with IL-6. Table 1 summarizes the pathological features in intestinal tissue after administration of IL-6 (200 μg/kg intraperitoneally). In comparison with control rats, in all IL-6-treated animals, except in rat 4, inflammatory cell infiltration (ranging from mild to severe) in the mucosa and submucosa with an increased number of polymorphonuclear cells was found. Furthermore, oedema was found in the sub-mucosa of the bowel of five experimental animals (Fig. 5). Five out of

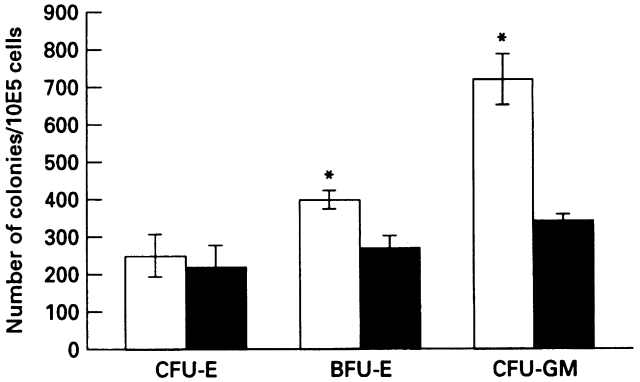

Fig. 2. Bone marrow of two experimental groups was cultured on day 14 as described in Materials and Methods. The number of colony-forming units-erythroid (CFU-E), burst-forming units-erythroid (BFU-E) and colony-forming units-granulocyte-macrophages (CFU-GM) was counted after 3, 7 and 11 days of culture for CFU-E, CFU-GM and BFU-E, respectively. Data are respresented as mean ± s.e.m. for colonies of three independent cultures, inside one group of experimental animals. *Different from control group ($P < 0{\cdot}05$). □, IL-6 group; ■, controls.

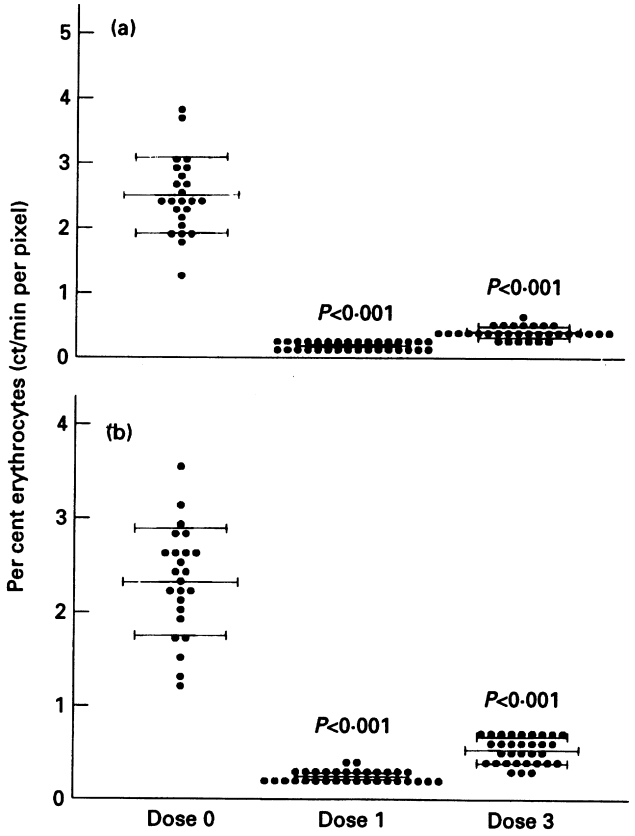

Fig. 3. Radioactivity of ^{33m}Tc erythrocytes (ct/min) was measured in heart (a) and liver (b) of the control rats (heart 0, liver 0) and in rats that were treated with one or three doses of IL-6 (200 μg/kg) (heart and liver 1 and 3, respectively). Data are presented as mean $\pm$ s.e.m. ct/min per pixel expressed as percentage of total body radioactivity as measured by gamma camera.

eight rats revealed minor to extensive bleeding in the intestinal wall. No differences in the structure of blood vessels were observed between IL-6-treated and control animals.

DISCUSSION

In order to elucidate the nature of IL-6-induced anaemia, the effects of IL-6 on haematological parameters were investigated in an experimental animal model. IL-6 was administered intraperitoneally in rats for 14 consecutive days. A decrease of Hb and Ht was observed, which was associated with a significant increase in reticulocyte number, leucocytes and platelets. No bone marrow suppression or sequestration of erythrocytes was found. It appeared that anaemia was caused by intestinal blood loss.

The results of present study are in accordance with previous observations, which showed the ability of IL-6 to induce anaemia in different animal models and in humans [12,15]. Thus, a relationship between IL-6 and anaemia seems to be established.

Although in some studies haemolysis was ruled out, other mechanisms such as modulation of iron metabolism, decreased bone marrow erythropoiesis and sequestration of erythrocytes, by which IL-6 may induce anaemia, remained uncertain.

In the present study IL-6 induced decreased plasma and bone marrow iron contents in the IL-6 group compared with the control

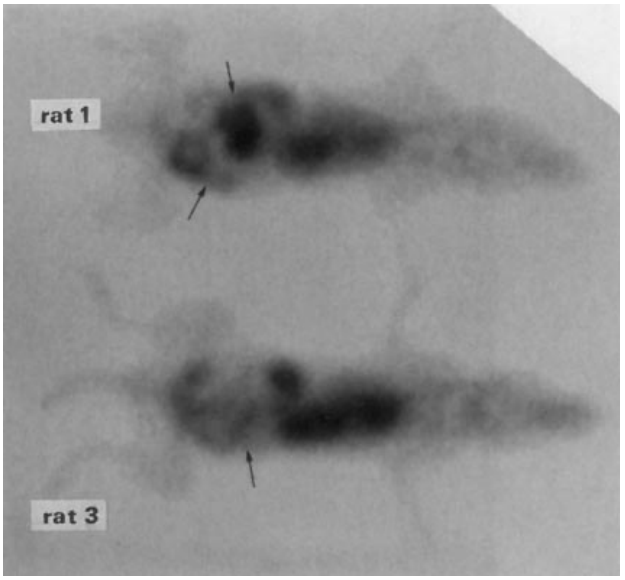

Fig. 4. Scintigram. The arrow indicates the accumulation of radioactivity (^{99m}Tc erythrocytes) in the abdominal part of the body of the IL-6 treated-rats.

group, suggesting a modulating effect of IL-6 on iron metabolism. IL-6 is a potent inducer of the synthesis of acute-phase proteins, such as ferritin [4], which may lead to iron retention in the intracellular space of the reticuloendothelial system, and a lower availability of iron needed for erythropoiesis [24]. However, in case of iron retention, such as is observed in ACD, increased amounts of iron in bone marrow are usually observed. In the present study anaemia appeared rapidly after starting the administration of IL-6, suggesting that other mechanisms than iron retention may be involved as well. Since intestinal blood loss was observed in the rats treated with IL-6, loss of iron may also have contributed to the development of iron deficiency and anaemia in this experimental model.

The present study does not provide evidence for suppression of erythropoiesis by IL-6. Previously, it has been shown that IL-6

Table 1. Intestinal histological changes due to administration of IL-6

		Intestinal changes		
Rat no.	Doses of IL-6	Bleeding	Oedema	Inflammatory infiltration
1	1	+++	++	+++
2	1	–	–	+
3	1	++	+	++
4	1	–	–	–
5	3	–	+	+
6	3	+	+	+
7	3	–	–	++
8	3	++	–	++

Tissues were evaluated for histological features.
–, No pathological changes; +, mild; ++, moderate; +++, severe histological changes.

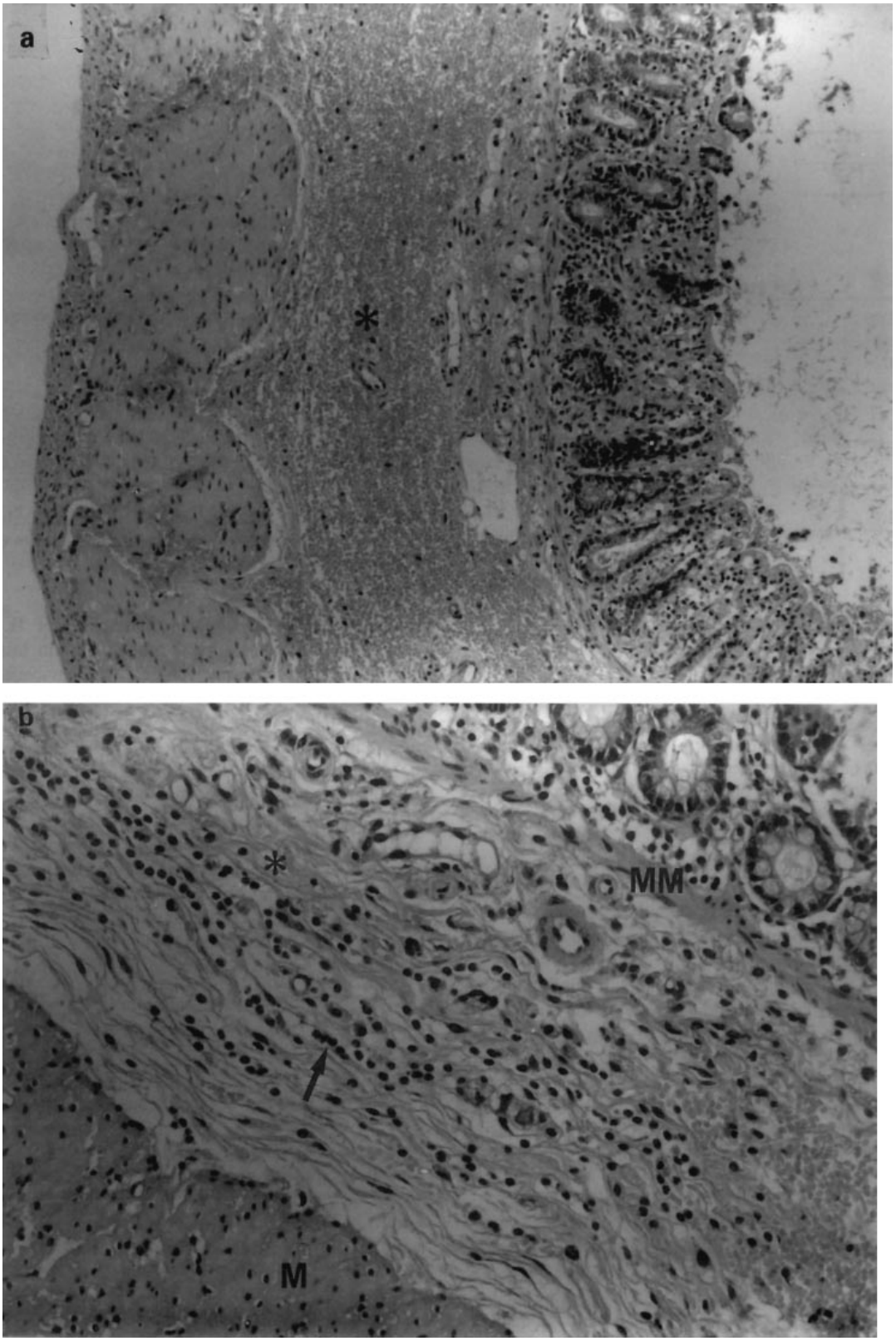

Fig. 5. Histological sections, stained with haematoxylin–eosin, from IL-6 injected rat (no. 1, caecum). (a) Extensive bleeding (*). (b) Submucosa (*): oedema and inflammatory cell infiltrate (arrow), MM, Muscularis mucosae; M, Muscularis.

exerts stimulative rather than suppressive effects on BFU-E [25], whereas addition of anti-IL-6 decreases BFU-E colony growth *in vitro* [11]. Similar to BFU-E, IL-6 causes an increase in the number of CFU-GM, leucocytes and platelets [26–28]. Our data are in accordance with those previous findings. Thus, suppression of erythropoiesis does not seem to play an important part in the pathogenesis of IL-6-induced anaemia.

Finally, sequestration of erythrocytes was mentioned as a possible explanation for IL-6 induced anaemia [12,15], whereas previously a shortened life span of erythrocytes was postulated to be involved in ACD [29]. No evidence for enhanced elimination of erythrocytes, from the circulation to the liver, was found after i.p. injection of IL-6 in the present study. However, the sequestration of erythrocytes in other organs, such as the spleen, has to be considered.

In this study it appeared that IL-6 was able to induce inflammatory cell infiltration, oedema and bleeding in the intestinal wall of rats treated with IL-6. Patton *et al*. [30] and others found similar histological intestinal changes due to administration of the TNF-α [31] and IL-1 [32] in rodents. Intestinal ulceration and inflammation as well as blood loss in patients with RA have been described. Side effects of medication, particularly non-steroid antiinflammatory drugs (NSAID), were thought to be the main cause of these pathological features. However, according to the results of the present study, the role of IL-6 has to be considered.

In conclusion, this study suggests that IL-6 can induce anaemia in rats. No evidence was found for suppressed erythropoiesis and erythrocyte sequestration in the IL-6-treated animals, whereas iron deficiency occurred during treatment with IL-6. Blood loss associated with intestinal inflammation during treatment with IL-6 appeared to be one of the mechanisms leading to anaemia in this experimental animal model. Whether there is a relationship between this experimental finding and the role of IL-6 in development of anaemia and/or intestinal inflammation in patients with RA still has to be clarified.

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