ANAEMIA OF CHRONIC DISEASE IN RHEUMATOID ARTHRITIS: IN VIVO EFFECTS OF TUMOUR NECROSIS FACTOR α BLOCKADE

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SUMMARY

Anaemia of chronic disease (ACD) is a common feature of active rheumatoid arthritis (RA). Inflammatory cytokines, particularly tumour necrosis factor α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), are thought to contribute to the pathogenesis of ACD, possibly by inhibiting erythropoietin (EPO) production. In this study, we examined the in vivo effects of TNF-α blockade with a chimeric monoclonal antibody, cA2, on erythropoiesis in RA patients with ACD. Administration of cA2 led to a dose-dependent increase in haemoglobin levels compared to placebo and these changes were accompanied by a reduction in both EPO and IL-6 levels. The data support the notion that TNF-α is important in the causation of ACD, but suggest a mechanism independent of EPO suppression. Instead, TNF-α may act directly on bone marrow red cell precursors.

KEY WORDS: Anaemia of chronic disease, Rheumatoid arthritis, Tumour necrosis factor α, Interleukin-6, Erythropoietin, Haemoglobin.

RHEUMATOID arthritis (RA) is associated with anaemia in up to 50% of patients [1], with iron deficiency and anaemia of chronic disease (ACD) being the most common causes. The pathogenesis of ACD has not been fully established and probably includes several mechanisms, including abnormalities of iron absorption [2] and release from macrophages [3], as well as dysregulation of the cytokine network. Erythropoietin (EPO) is the major physiological growth factor for red blood cell development and studies show that although EPO levels are raised in anaemic RA patients, they are not as high as in equally anaemic iron-deficient controls [4–6], suggesting that there is deficient EPO production in RA. This may be related to the finding that altered intracellular iron balance in ACD has been shown to regulate EPO production both in vitro and in vivo, possibly via modulation of the hypoxia-sensitive haem protein which is thought to regulate EPO gene expression [7]. Reports of successful treatment of ACD with recombinant EPO therapy in RA [8–11], as well as chronic renal disease [12, 13] and inflammatory bowel disease [14], support this notion. However, patients with more active rheumatoid disease show a slower improvement in haemoglobin levels in response to recombinant human (rh) EPO [15], suggesting that other factors may also be important.

There is growing evidence to suggest that inflammatory cytokines, particularly tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interferon-γ [16, 17], contribute to ACD in RA. TNF-α has been shown to inhibit macrophage iron release in vitro [18], and animals treated with TNF-α develop anaemia with a low serum iron and preserved iron stores [19]. TNF-α also inhibits the formation of early red cell colonies in bone marrow cultures in vitro [20–22] and in vivo [19]. Studies using mice deficient for either the p55 or the p75 kDa TNF receptors have indicated that this effect is mediated principally by the p55 TNF receptor, possibly through inhibition of cycling of red cell precursors from the G1/S phase [23]. The reduction in erythroid colony formation can be partly overcome in vivo by adding excess EPO to the culture system, resulting in a dose-dependent restoration of red cell colony numbers [11]. In vivo, EPO administration prevents some of the suppressive effects seen on red cell formation in TNF-α-treated mice, also in a dose-dependent fashion [24]. Serum levels of TNF-α have been shown to correlate inversely with haemoglobin (Hb) as well as parameters of disease activity in RA patients with ACD [25, 26]. These findings suggest that TNF-α may contribute to ACD by reducing the sensitivity of erythroblasts to EPO.

IL-6 also has diverse haematological effects and causes reversible anaemia when administered in vivo, both in animal models [27], and following therapy for malignancies in humans [28, 29]. The underlying mechanism has not been fully established, although a haemodilutional effect has been postulated [30]. Circulating IL-6 levels have been shown to be highest amongst RA patients with ACD [31], although this may be a reflection of disease activity rather than implying a direct effect of IL-6 on erythropoiesis. IL-1, like TNF-α, has a pro-inflammatory role in RA and has been shown to inhibit red cell colony formation both in vitro [32] and in vivo; its effects can be prevented by the simultaneous administration of EPO [33, 34]. Bone marrow macro-
phages do not appear to be the major site of production of IL-1 and TNF-α in ACD, as removal of CD14-positive cells (macrophages) from the in vitro marrow culture system does not restore red cell numbers [35].

We have previously reported [36] that RA patients treated with a chimeric antibody to TNF-α, cA2, showed significant improvements relative to placebo in disease activity measures such as joint score and C-reactive protein (CRP). In addition, we saw improvements in Hb levels in cA2-treated patients, despite repeated venesection. The aim of this study was to investigate the mechanism underlying the recovery from ACD in patients treated with cA2. In particular, we focused on circulating EPO, and related changes in this growth factor to changes in Hb in RA patients with ACD.

MATERIALS AND METHODS

Study design

Details have been published previously [36]. Briefly, 73 patients were enrolled in a double-blind placebo-controlled trial of a chimeric monoclonal antibody to TNF-α (cA2, IgG1 isotype). All patients had active RA [defined as at least six swollen joints as well as 3/4 of the following: six or more tender/painful joints, early morning stiffness 45 min, erythrocyte sedimentation rate (ESR) 28 mm/h, CRP ≥ 20 mg/l]. All patients had had RA for at least 6 months (fulfilling the ACR criteria [37]), and had failed at least one disease-modifying agent. Patients were randomly assigned to receive single infusions of either cA2 (1 or 10 mg/kg) or placebo (human albumin).

Sample collection

Peripheral blood samples from 64 of these patients were used for the purpose of this study, with venesection being performed immediately prior to infusion (week 0, mean time 08:30 a.m.), and at 2 and 4 weeks post-infusion (mean time of blood sampling 10:45 a.m.). In two patients, 2 week samples were unavailable and so week 1 samples were substituted. In two different patients, samples 3 weeks post-infusion were used instead of 4 weeks, for the same reason. For the preparation of serum, blood was collected into sterile tubes, allowed to clot for 30 min and spun at room temperature for 20 min at 2500 g. Serum samples were incubated in microtitre plates pre-coated with a monoclonal antibody to EPO and bound EPO was detected using an enzyme-linked polyclonal antibody specific for EPO. Optical density values obtained at 450 nm were compared to those obtained for standards covering the range 2.5–200 mIU/ml, with the normal range for this assay being 3.5–16.6 mIU/ml.

IL-6 was measured by an enzyme amplified sensitivity immunoassay technique, based on an oligoclonal detection system (Medgenix Diagnostics, Brussels, Belgium). Briefly, serum samples were incubated in microtitre plates pre-coated with a cocktail of monoclonal antibodies to IL-6 and bound cytokine was detected by the addition of complementary monoclonal antibodies to IL-6, conjugated to horseradish peroxidase. Optical density values obtained at 450 nm were compared to those obtained for a series of standards covering the range 10–2000 pg/ml, the normal range being <10 pg/ml.

A quantitative sandwich enzyme immunoassay was used to measure serum ferritin (Cambridge Life Sciences, UK). Briefly, serum samples were incubated with monoclonal antibody-coated micropins specific for human liver ferritin. Bound ferritin was detected by incubation with complementary anti-ferritin antibodies conjugated to horseradish peroxidase. Optical density values obtained at 450 nm were compared to those for standards covering the range 15–500 ng/ml, the normal range for females being 6–120 ng/ml and that for males 10–400 ng/ml.

Haemoglobin concentrations were measured using a Coulter counter (Coulter, USA) according to the manufacturer’s recommended procedure. The normal range of Hb for females was 12–16 g/dl and that for males 13.5–17.5 g/dl.

Analysis of results

In order to exclude iron deficiency as a confounding factor, we identified patients who had co-existing iron deficiency and ACD by using baseline serum ferritin levels as an indirect measure of iron stores. As ferritin is an acute-phase protein, higher levels are compatible with iron deficiency in rheumatoid patients than in patients without inflammatory disease. In RA patients, a serum ferritin level of <60 ng/ml has been shown to be a reliable indicator of iron deficiency with a high degree of sensitivity [38, 39]. Patients with pre-infusion ferritin levels of <60 ng/ml were therefore excluded from analysis.

The placebo, and 1 and 10 mg/kg cA2 groups were compared using the Mann–Whitney U-test. Spearman’s ranked coefficient of correlation was used to assess the relationship between different parameters. In view of the inclusion of both males and females in this study and the difference in normal Hb values according to sex, analysis was performed on the change from baseline.
RESULTS

Baseline (pre-infusion) demographic and laboratory data for the three treatment groups are shown in Table I. Sixteen of the 64 patients studied had a baseline ferritin of <60 ng/ml, indicating iron deficiency [38], and were excluded from further analysis. Of the 48 remaining patients, 33 had Hb concentrations below the normal range for their sex and were classified as having ACD. Median circulating EPO levels were within the normal range for all three groups at baseline, although 13 of the 48 individual values were elevated. Median circulating IL-6 values were high in all groups at baseline, with all but three patients showing values above the normal range (Table I and data not shown). There were no significant differences between the three treatment groups for any laboratory measurement at baseline.

Changes in Hb, EPO and IL-6 over the course of the study are summarized by treatment group in Table II. Patients treated with high-dose cA2 showed a small but significant improvement in Hb in the 4 weeks following infusion, most marked at week 2, while the placebo group showed a larger reduction (Fig. 1). Patients treated with low-dose cA2 showed maintenance of baseline Hb. The significance of the changes in median Hb is underlined by an analysis of the proportion of patients who had ACD before and after treatment: 87% of patients treated with high-dose cA2 had ACD at the start of the study, compared with 73% 4 weeks after infusion. In contrast, the percentage of ACD patients in the placebo group rose from 64 to 86% over the same time period.

EPO levels rose in the placebo group at 2 and 4 weeks post-infusion, but fell in the 10 mg/kg cA2-treatment group at 2 weeks, reaching statistical significance compared to the placebo-treated patients (P < 0.05) (Fig. 2). IL-6 levels also fell significantly in both cA2-treated groups compared to placebo (P < 0.001 at 2 weeks; Fig. 3), as previously fully reported [40]. After pooling the results for all three treatment groups, there was a significant inverse relationship between Hb and EPO levels at baseline (r = 0.575, P < 0.001; Fig. 4) and between the change from baseline in Hb and EPO at 2 weeks (r = 0.549, P < 0.001; Fig. 5). There were no significant differences between the three treatment groups for any laboratory measurement at baseline.

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### Table I

Baseline characteristics of patients

<table>
<thead>
<tr>
<th>Dose of cA2</th>
<th>Placebo</th>
<th>1 mg/kg</th>
<th>10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patients</td>
<td>n = 20</td>
<td>n = 23</td>
<td>n = 21</td>
</tr>
<tr>
<td>Female/male</td>
<td>16/4</td>
<td>18/5</td>
<td>17/4</td>
</tr>
<tr>
<td>Ferritin &lt;60 ng/ml</td>
<td>6</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Hb (g/dl)*</td>
<td>11.9 (9.9, 12.6)†</td>
<td>11.7 (10.2, 13.0)</td>
<td>11.0 (10.6, 12.0)</td>
</tr>
<tr>
<td>EPO (mIU/l)*</td>
<td>9.3 (4.7, 24.5)</td>
<td>9.0 (6.6, 20.5)</td>
<td>10.5 (6.6, 22)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)*</td>
<td>155.6 (67.0, 219.7)</td>
<td>173.5 (54.0, 241.3)</td>
<td>115 (78.0, 203)</td>
</tr>
</tbody>
</table>

Hb, haemoglobin; EPO, erythropoietin; IL-6, interleukin-6. Normal ranges: Hb 12–16 g/dl (females), 13.5–17.5 g/dl (males); EPO 3.3–16.6 mIU/l; IL-6 < 10 pg/ml.
*Excluding patients with baseline ferritin <60 ng/ml.
†Median (interquartile range).

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**Fig. 1.**—Change in haemoglobin (Hb) at 2 and 4 weeks post-infusion compared to baseline levels in the three different treatment groups. ***P < 0.01; ****P < 0.001; Mann–Whitney U-test.

**Fig. 2.**—Change in erythropoietin (EPO) at 2 and 4 weeks post-infusion compared to baseline levels in the three different groups. *P < 0.05; Mann–Whitney U-test.
DISCUSSION

The results of this study are consistent with earlier reports [1] in demonstrating a high incidence of anaemia in RA. Approximately 60% of our study group were anaemic at baseline and ACD was found to account for 67% of these, emphasizing the relative importance of this condition in RA.

Our data show that cA2 therapy not only improves parameters of disease activity such as joint scores, pain and CRP [36], but also helps to correct ACD. Although the improvement in median Hb following cA2 administration was small in absolute terms, it took place over a short time period (4 weeks), occurred despite a significant venesection (0–500 ml) and contrasted with a larger fall in median Hb in patients treated with placebo. The difference between the treatment groups was highly statistically significant.

Although the improvement in Hb seen in cA2-treated patients may reflect the reduction in their disease activity, an alternative explanation is that cA2 inhibits a direct effect of TNF-α on erythropoiesis. By measuring circulating cytokines in these patients, we were able to test the hypothesis that TNF-α contributes to ACD in RA by inhibiting EPO production. This notion would predict a rise in circulating EPO following cA2 administration in vivo, with no change or even reciprocal changes in placebo patients. In the event, however, we saw the opposite, with a fall in EPO in cA2-treated patients and a rise in those receiving placebo.

These findings suggest that the major effect of TNF-α in ACD is not through inhibition of EPO production and that, instead, TNF-α may act directly to inhibit red cell formation in the bone marrow. Anaemia is known to be a major stimulus of EPO, and is thought to regulate its levels via a feedback control system [41]. Such a regulatory mechanism could explain our findings that improve-

| TABLE II |
| Change in Hb, EPO and IL-6 post-infusion compared to baseline levels |

<table>
<thead>
<tr>
<th>Week 2</th>
<th>Placebo</th>
<th>1 mg/kg</th>
<th>10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 14</td>
<td>n = 19</td>
<td>n = 15</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>−0.5 (−0.83, −0.1)†</td>
<td>0 (−0.8, 0.4)</td>
<td>0.2*** (0, 0.5)</td>
</tr>
<tr>
<td>EPO (mIU/ml)</td>
<td>3.6 (1.17, 10.1)</td>
<td>0.4 (−1.6, 4.7)</td>
<td>−2.6* (−5.0, 3.0)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>10 (−39.5, 123.5)</td>
<td>−133*** (−182.5, −26.8)</td>
<td>−105*** (−175, −62.5)</td>
</tr>
<tr>
<td>Week 4</td>
<td>Hb (g/dl)</td>
<td>−0.5 (−0.93, 0)</td>
<td>−0.1 (−1.0, 0.7)</td>
</tr>
<tr>
<td>EPO (mIU/ml)</td>
<td>1.3 (−0.1, 8.5)</td>
<td>−0.1 (−3.0, 2.0)</td>
<td>0.2 (−1.5, 5.5)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>−12 (−63.7, 34.7)</td>
<td>−63 (−162.5, 6.5)</td>
<td>−79* (−142.0, −52.0)</td>
</tr>
</tbody>
</table>

Hb, haemoglobin; EPO, erythropoietin; IL-6, interleukin-6. Normal ranges: Hb 12–16 g/dl (females), 13.5–17.5 g/dl (males); EPO 3.3–16.6 mIU/l; IL-6 < 10 pg/ml.

†Median change

Fig. 3.—Change in interleukin-6 (IL-6) at 2 and 4 weeks post-infusion compared to baseline levels in the three different groups. *P < 0.05; ***P < 0.001; Mann–Whitney U-test.

Fig. 4.—Correlation between baseline haemoglobin (Hb) and erythropoietin (EPO) levels using data from all three treatment groups. Each point represents an individual patient. *P < 0.001; Spearman’s ranked correlation coefficient.
ment in Hb following cA2 therapy was accompanied by a reduction in EPO levels and vice versa in the placebo group.

Although this study cannot address the question of how TNF-α acts in the marrow, it is likely that it causes a reduction in the sensitivity of erythroblasts to EPO. The finding that administration of rhEPO can successfully treat ACD in a variety of clinical situations indicates that this relative insensitivity to EPO can be overcome, provided adequate EPO concentrations are maintained. The rate of response to rhEPO appears to be determined by the degree of RA disease activity and inflammation [15], which in turn may reflect TNF-α production. Alternatively, cA2 may prevent the inhibitory effects of TNF-α on erythroid progenitor cell cycling [23].

cA2 therapy may also improve ACD indirectly by reducing levels of other cytokines thought to play a suppressive role in erythropoiesis, such as IL-6. In support of this, we saw a strong inverse relationship between changes in IL-6 and Hb following cA2.

The site of production of TNF-α relevant to ACD remains to be established, but evidence suggests that the principal source is not the bone marrow macrophages [35]. Raised levels of TNF-α, its receptors and TNF-α mRNA have all been detected within the RA synovial joint [42–44], where there is strong evidence that TNF-α contributes to the development of arthritis [45, 46]. The synovial joint would therefore appear to be the most likely source of TNF-α, circulating levels producing a systemic effect on the bone marrow in ACD.

Improvement in ACD following high-dose oral corticosteroid treatment in RA has been reported recently [47]. Interestingly, Hb showed significant improvement as early as 4 days after prednisolone therapy was instituted and was accompanied by a fall in EPO levels, indicating a mechanism other than increased EPO production. Corticosteroids have been shown to downregulate the production of various inflammatory cytokines, such as TNF-α [48] and IL-1 [49], raising the possibility that the pro-erythropoietic actions of these drugs may also depend on inflammatory cytokine inhibition.

Improvement of anaemia in RA patients may be of direct benefit, e.g. by reducing fatigue [15], and so increasing the ability to cope with everyday activities. Anaemia also contributes to more serious conditions such as cardiac failure, of particular relevance in view of the greater risk of cardiovascular disease in RA patients [50]. Other benefits may accrue from interference by cA2 in disordered erythropoiesis in RA: a greater ability to mobilize and incorporate iron from storage sites and macrophages into red cells may reduce iron-associated free radical formation [51] and thereby indirectly reduce articular disease.

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REFERENCES


![Graph](image)


