RECOMBINANT HUMAN ERYTHROPOIETIN IN THE TREATMENT OF RENAL ANAEMIA

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for the degree of Doctor of Medicine

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1. CONTENTS

2. INDEX OF TABLES

Chapter 5

Chapter 6

Chapter 7

3. INDEX OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

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5. SUMMARY

This thesis is concerned with a study of the clinical and biological effects of recombinant human erythropoietin (EPO) which recently became available as a novel treatment for the anaemia of end-stage renal disease. The dissertation begins with an introductory chapter which contains a historical note and survey of the relevant published literature. In this section is included a review of the pathogenesis of renal anaemia, the traditional treatment options for this condition, the development of recombinant human EPO, the biochemistry and physiology of erythropoietin, the metabolic fate of EPO, and the early clinical trials with EPO. This is followed by seven chapters of original work undertaken in Cardiff between April 1988 and July 1990:-

Chapter 1 Pharmacokinetics of recombinant human erythropoietin

This chapter reports a study comparing the single-dose pharmacokinetics of intravenously-, intraperitoneally-, and subcutaneously-administered EPO in 8 CAPD patients, with a view to determining the optimal dosage regimen and route of administration for the drug. Following IV administration, serum EPO levels decayed mono-exponentially with a mean half-life of 8.2 hours, and only 2.3% of the administered dose was lost in the peritoneal dialysis fluid. With IP administration, peak serum levels were attained at 12 hours which were only 9% of those obtained following IV EPO despite a six-fold larger dose. Using the same dose as that administered IV, SC-administered EPO yielded peak levels of only 4% of those obtained after IV EPO, 18 hours following the injection. The bioavailability of IP EPO was only 2.9%; that of SC EPO was seven times greater at 21.5%. Because of this, and the fact that serum levels remained above endogenous levels up to 96 hours, it was concluded that SC administration of EPO was likely to be the optimal route for treating dialysis patients. Simulated multiple-dose pharmacokinetic analysis suggested that a twice- or thrice-weekly dosage regimen would be practicable.

Chapter 2 Haematological response to recombinant human erythropoietin in patients with renal anaemia

This study monitored the haemoglobin concentration, red cell indices, reticulocyte count, and iron metabolism in 3 groups of dialysis patients treated with EPO:-

Group 1: 10 haemodialysis patients treated with IV EPO 240 U/kg/wk.

Group 2: 15 CAPD patients treated with SC EPO 120 U/kg/wk.

Group 3: 13 haemodialysis patients treated with SC EPO 120 U/kg/wk.

In all 3 groups there was an early increase in the reticulocyte count followed by a gradual rise in the haemoglobin concentration from around 6 g/dl to a target level of 10-12 g/dl over a period of about 12-16 weeks. The serum ferritin fell dramatically, and 26 of the 38 patients required intravenous iron supplementation to maintain adequate iron supplies. A large variation in the maintenance doses required to keep the haemoglobin within the target range was observed, but generally lower doses were needed when given SC compared to IV administration. Examples were seen of inhibition of the response to EPO by infection and malignancy.

Ferrokinetic and blood volume studies were performed in 12 of the CAPD patients. A two- to three-fold increase in marrow erythropoietic activity was found after EPO therapy, and the rise in red cell mass was associated with a compensatory fall in plasma volume such that the circulating whole blood volume remained unchanged.

Chapter 3 Cardiorespiratory benefits following correction of renal anaemia by erythropoietin

A number of parameters of cardiorespiratory function were monitored in 10 haemodialysis patients during the first 12 months of EPO therapy. There were considerable improvements in exercise capacity, maximal oxygen consumption, and anaerobic threshold (measured by respiratory gas analysis during treadmill exercise

testing) after 2 months' treatment, and these increases were maintained but not further augmented on repeat testing at 4, 8, and 12 months. The carbon monoxide transfer factor at rest also showed a dramatic improvement after EPO. In addition there was a substantial reduction in exercise-induced myocardial ischaemia following treatment (8 of 10 patients had significant S-T segment depression on exercise ECG before EPO, 1 of 10 after 2 months, and 0 of 9 after 12 months of EPO). Left ventricular mass, as assessed by echocardiography, progressively decreased over the first 12 months of treatment, and 4 of 9 patients showed a significant reduction in cardiac size on chest X-ray. Thus, this study showed considerable long-term improvements in cardiorespiratory function after EPO therapy.

Chapter 4 Rheological studies during treatment of renal anaemia with erythropoietin

Whole blood, plasma, and serum viscosities together with red cell deformability were measured before and during EPO treatment in order to characterise the possible role of rheological changes in the pathogenesis of side-effects such as hypertension and arteriovenous fistula thrombosis associated with this drug. Whole blood viscosity progressively increased during the first 4 months of treatment, and this increase was exponential for a linear increase in haemoglobin concentration. There was no change in either plasma or serum viscosity or in red cell deformability during this period. Detailed analysis of the relationship between the viscosity changes and the haemoglobin concentration indicated that the rheological changes which occur with correction of anaemia by EPO can be attributed solely to the increase in circulating red cell mass rather than to any changes in the properties of the plasma or the erythrocytes themselves.

Chapter 5 Fistula blood flow and coagulation studies during erythropoietin therapy

This study was prompted by the reports of an increased incidence of arteriovenous fistula thrombosis in haemodialysis patients treated with EPO. Fistula blood flow (measured by Doppler ultrasound) and a variety of tests of coagulation and haemostasis were monitored in 10 haemodialysis patients receiving EPO therapy. Fistula blood flow did not significantly change during the first 12 months of EPO. Bleeding time improved in all 10 patients after 4 months of treatment, and this improvement was maintained at 12 months. There were no significant changes in one-stage prothrombin time, kaolin cephalin clotting time, whole blood clotting time, prothrombin consumption index, plasma fibrinogen, factor VII, factor VIII, anti-thrombin III levels, or platelet aggregability to ADP after EPO therapy. In contrast, there was a progressive reduction in protein C and protein S levels over the first 4 months, which reverted to pre-treatment values by 8 and 12 months. Since the levels of protein C and protein S attained at 4 months are known to predispose to thrombosis, it was interesting to speculate that this effect may contribute to the high incidence of fistula thrombosis observed in EPO-treated haemodialysis patients.

Chapter 6 Studies using ^{125}I-labelled recombinant human erythropoietin

In this chapter, the use of ^{125}iodine-labelled EPO as a tool for examining the absorption kinetics and the metabolic fate of EPO is reported. The chloramine-T method was used to radio-iodinate carrier-free recombinant EPO. Gel permeation chromatography and SDS-polyacrylamide gel electrophoresis were used to characterise the nature of the radiolabelled material, and its bioactivity was confirmed by the polycythaemic mouse bioassay. The kinetics and clearance of IV-administered ^{125}I-EPO were studied in 6 normal healthy volunteers, and the reasons for discrepancies with the results from pharmacokinetic studies using unlabelled EPO are discussed. Studies in one

subject failed to identify any smaller molecular weight metabolites of EPO in circulation.

In a separate study, the absorption kinetics and bioavailability of ^{125}I-EPO injected into 3 different subcutaneous sites in 8 normal healthy volunteers were examined. Injection into the thigh resulted in more rapid absorption, higher peak serum levels, greater bioavailability, and less drug remaining at the SC site after 96 hours compared with injection into the arm or abdomen. These results suggested that the optimal site for SC administration of EPO may be the thigh.

Chapter 7 Treating renal anaemia with recombinant human erythropoietin: practical guidelines and a clinical algorithm

This chapter contains a series of guidelines which were compiled on the basis of the experience gained by the author in treating 42 patients in Cardiff Royal Infirmary between 1988 and 1990. In addition to providing an algorithm which focuses particularly on the requirements for iron supplementation, the discussion deals with such issues as selection of patients for EPO therapy, the optimal starting dose and route of administration, the monitoring of iron status during treatment, the desirable rate of response and target haemoglobin concentration, and the investigation of an impaired response, as well as potential complications. Much of the work contained in this thesis has contributed to this set of recommendations, which also reflects the current state of knowledge and opinion from the published literature.

6. INDEX OF PUBLICATIONS

The following is a list of publications arising from the work presented in this thesis:-

Chapters

1. **Macdougall IC**, Stevens JM, Hughes R, Hutton RD, Coles GA, Williams JD. Recombinant human erythropoietin (epoetin alfa and beta) in patients on ambulatory peritoneal dialysis. In: Adamson JW, Erslev AJ, Eschbach JW, Winearls CG, eds. Erythropoietin: Molecular, Cellular, and Clinical Biology, Ch 18. The John Hopkins University Press, Baltimore and London (in press).

Papers

1. **Macdougall IC**, Roberts DE, Neubert P, Dharmasena AD, Coles GA, Williams JD. Pharmacokinetics of recombinant human erythropoietin in patients on continuous ambulatory peritoneal dialysis. Lancet 1989; i: 425-427.

2. **Macdougall IC**, Hutton RD, Cavill I, Coles GA, Williams JD. Poor response to treatment of renal anaemia with erythropoietin corrected by iron given intravenously. Br Med J 1989; 299: 157-158.

3. **Macdougall IC**, Roberts DE, Neubert P, Dharmasena AD, Coles GA, Williams JD. Pharmacokinetics of intravenous, intraperitoneal, and subcutaneous recombinant erythropoietin in patients on CAPD: a rationale for treatment. Contrib Nephrol 1989; 76: 112-121.

4. **Macdougall IC**, Cavill I, Davies ME, Hutton RD, Coles GA, Williams JD. Subcutaneous recombinant erythropoietin in the treatment of renal anaemia in CAPD patients. Contrib Nephrol 1989; 76: 219-226.

5. **Macdougall IC**, Lewis NP, Saunders MJ, Cochlin DL, Davies ME, Hutton RD, Fox KAA, Coles GA, Williams JD. Long-term cardiorespiratory effects of amelioration of renal anaemia by erythropoietin. Lancet 1990; 335: 489-493.

6. **Macdougall IC**, Hutton RD, Cavill I, Coles GA, Williams JD. Treating renal anaemia with recombinant human erythropoietin: practical guidelines and a clinical algorithm. Br Med J 1990; 300: 655-659.

7. **Macdougall IC**, Hutton RD, Cavill I, Coles GA, Williams JD. Recombinant human erythropoietin in the treatment of renal anaemia - an update. Nefrologia 1990; 10(suppl. 2): 23-32.

8. **Macdougall IC**, Davies ME, Hutton RD, Cavill I, Lewis NP, Coles GA, Williams JD. The treatment of renal anaemia in CAPD patients with recombinant human erythropoietin. Nephrol Dial Transplant 1990; 5: 950-955.

9. **Macdougall IC**, Hutton RD, Coles GA, Williams JD. The use of erythropoietin in renal failure. Postgrad Med J 1991; 67: 9-15.

10. **Macdougall IC**, Roberts DE, Coles GA, Williams JD. Clinical pharmacokinetics of epoetin (recombinant human erythropoietin). Clin Pharmacokinet 1991; 20: 99-113.

11. **Macdougall IC**, Jones JM, Robinson MI, Miles JB, Coles GA, Williams JD. Subcutaneous erythropoietin therapy: comparison of three different sites of injection. Contrib Nephrol 1991; 88: 152-156.

12. **Macdougall IC**, Roberts DE, Neubert P, Coles GA, Williams JD. Pharmacokinetics of recombinant human erythropoietin and its use in treating CAPD patients. Kidney Int (in press).

13. **Macdougall IC**, Davies ME, Hutton RD, Coles GA, Williams JD. Rheological studies during treatment of renal anaemia by recombinant human erythropoietin. Br J Haem (in press).

14. Winearls CG, **Macdougall IC**. Epoetin therapy in the treatment of renal anaemia. Eur J Clin Pharm (in press).

15. **Macdougall IC**, Cavill I, Hutton RD, Coles GA, Williams JD. Clinical recognition of functional iron deficiency in chronic dialysis patients. Semin Dial (in press).

Papers in preparation

16. **Macdougall IC**, Davies ME, McLellan D, Cochlin DL, Hutton RD, Coles GA, Williams JD. Fistula blood flow, blood viscosity, and protein C and S levels during erythropoietin therapy in haemodialysis patients.

17. **Macdougall IC**, Trevett D, Jones EA, Cavill I, Evans WD, Coles GA, Williams JD. Factors influencing the haematological response to recombinant erythropoietin in dialysis patients.

18. Lewis NP, **Macdougall IC**, Willis N, Henderson AH. The ventilatory cost of exercise compared in chronic heart failure and chronic renal anaemia.

19. Lewis NP, **Macdougall IC**, Willis N, Coles GA, Williams JD, Henderson AH. Effects of the partial correction of renal anaemia with recombinant human erythropoietin on exercise physiology.

20. **Macdougall IC**, Cavill I, Hulme B, Bain B, McGregor E, McKay P, Coles GA, Williams JD. Detection of functional iron deficiency during epoetin therapy: a new approach.

Letters

1 **Macdougall IC**, Roberts DE, Coles GA, Williams JD. Intraperitoneal erythropoietin. Lancet 1989; i: 1389.

2. **Macdougall IC**, Hutton RD, Cavill I, Coles GA, Williams JD. Poor response to erythropoietin. Br Med J 1989; 299: 620-621.

Abstracts

1. **Macdougall IC**, Coles GA, Williams JD. The pharmacokinetics of recombinant erythropoietin in CAPD patients. Kidney Int 1989; 35: 273.

2. **Macdougall IC**, Roberts DE, Dharmasena AD, Coles GA, Williams JD. The pharmacokinetics of intravenous and intraperitoneal recombinant erythropoietin in CAPD patients. Nephrol Dial Transplant 1989; 4: 318.

3. **Macdougall IC**, Lewis NP, Saunders MJ, Cochlin DL, Davies ME, Hutton RD, Coles GA, Williams JD. Exercise capacity, fistula blood flow, and rheological studies during treatment with rHuEPO in haemodialysis patients. Nephrol Dial Transplant 1989; 4: 319.

4. **Macdougall IC**, Roberts DE, Dharmasena AD, Coles GA, Williams JD. The pharmacokinetics of IV and IP recombinant erythropoietin in CAPD patients. Perit Dial Int, IX Annual CAPD Abstracts, 108.

5. **Macdougall IC**, Lewis NP, Fox KAA, Coles GA, Williams JD. Increased exercise capacity and reversal of exercise-induced myocardial ischaemia after treatment with recombinant erythropoietin. EDTA Abstracts, Gothenburg 1989; 207.

6. **Macdougall IC**, Davies ME, Hutton RD, Coles GA, Williams JD. Reduction in protein C and protein S levels after treatment with recombinant erythropoietin. Nephrol Dial Transplant 1989; 4: 476.

7. Lewis NP, **Macdougall IC**, Coles GA, Williams JD, Fox KAA. Increased exercise capacity and reversal of exercise-induced myocardial ischaemia in haemodialysis patients treated with recombinant human erythropoietin. Br Heart J 1989; 61: 436.

8. Cavill I, Bentley NJ, **Macdougall IC**. Quantifying erythropoiesis in renal anaemia and erythropoietin therapy. Blood 1989; 74 (suppl.): 970.

9. Lewis NP, **Macdougall IC**, Coles GA, Williams JD, Fox KAA. Reversal of exercise-induced myocardial ischaemia and LVH, and improved exercise capacity in anaemic haemodialysis patients treated with recombinant human erythropoietin. Eur Heart J 1989; 10: 2269.

10. Williams JD, **Macdougall IC**, Davies ME, Hutton RD, Coles GA. Recombinant erythropoietin treatment is accompanied by a reduction in protein C and protein S levels. Kidney Int 1990; 37: 381.

11. Coles GA, **Macdougall IC**, Jones J, Davies M, Williams JD. The metabolism of ^{125}I-labelled recombinant human erythropoietin in man. Kidney Int 1990; 37: 366.

12. **Macdougall IC**, Jones JM, Robinson MI, Coles GA, Williams JD. Subcutaneous erythropoietin therapy: comparison of 3 different sites of injection. Nephrol Dial Transplant 1990; 5: 759.

13. **Macdougall IC**, Cavill I, Hulme B, Bain B, McGregor E, McKay P, Coles GA, Williams JD. Detection of functional iron deficiency during EPO therapy: a new approach. J Amer Soc Nephrol 1990; 1: 402.

14. Hutton RD, **Macdougall IC**, Davies ME, Coles GA, Williams JD. Rheological studies during treatment of renal anaemia by recombinant human erythropoietin. J Amer Soc Nephrol 1990; 1: 400.

15. Jones JM, **Macdougall IC**, Robinson MI, Coles GA, Williams JD. Subcutaneous erythropoietin therapy: comparison of 3 different sites of injection. J Amer Soc Nephrol 1990; 1: 400.

7. INTRODUCTION

The advent of recombinant human erythropoietin (EPO) represents a major breakthrough in the management of patients suffering from the anaemia of end-stage renal disease [1,2,3,4]. Prior to its availability a few years ago, many patients with renal anaemia either had to endure the disabling symptoms and morbidity associated with this condition, or run the risks of side-effects and complications inherent in the only available treatment options, namely androgen therapy or blood transfusions. Androgens tended to be beneficial only when the anaemia was mild, and were relatively ineffective in severe cases [5,6,7]. The side-effects of virilisation and hepatotoxicity also precluded their widespread usage [6,7]. Thus, many patients had to rely on regular blood transfusions, the effects of which were not only transient, but were associated with the risks of transmission of infectious agents (particularly viral), sensitisation to histocompatibility antigens, iron overload, transfusion reactions, and possible suppression of endogenous erythropoietin production.

It had been recognised for some time, however, that although the pathogenesis of renal anaemia was multifactorial, the major factor was a relative deficiency of erythropoietin [8]. Thus, patients with renal anaemia generally had circulating levels of erythropoietin which were inappropriately low for the degree of anaemia, in comparison to those with non-renal causes of anaemia such as iron deficiency [9]. It was, therefore, logical to treat renal anaemia with replacement erythropoietin. Until recently, however, the technology for synthesising this hormone was lacking, mainly due to the problems of isolating material which was present in only picomolar amounts in biological fluids. With the advent of recombinant DNA technology, however, this feat was finally achieved, thus providing a new therapeutic option in the management of anaemia associated with end-stage renal disease.

8. LITERATURE SURVEY: RENAL ANAEMIA

This literature survey will review various aspects of renal anaemia including its pathogenesis and traditional treatment options, the development of recombinant human EPO along with the biochemistry, physiology, and metabolic fate of the hormone, and finally the early clinical trials of EPO which occurred prior to the commencement of this project. Subsequent publications relevant to this subject will be discussed in each of the seven chapters of the thesis as appropriate.

1. Pathogenesis of renal anaemia

Anaemia is an almost invariable consequence of progressive renal failure [8,10,11]. The association was first documented by Richard Bright more than 150 years ago. The nature of this anaemia was, however, poorly understood until 30 years ago when Erslev [12] proved that erythropoietin was the hormone which regulated erythropoiesis, and Jacobson et al. [13] demonstrated that EPO originated in the kidney. The anaemia previously was thought to be due to the effects of uraemia, since its severity was roughly proportional to the degree of renal impairment [14], being particularly marked in anephric individuals. The anaemia is hypoproliferative in nature, as evidenced by a low reticulocyte count, a reduced ratio of erythroid to granulocytic elements in the marrow, and decreased marrow iron turnover as assessed by quantitative ferrokinetics [8]. The red cells, although reduced in number, are usually normochromic and normocytic unless there are superadded problems such as iron deficiency or aluminium intoxication [8,10,11].

A number of factors have been proposed as being contributory to the pathogenesis of renal anaemia. These include shortened red cell survival [15,16,17], inhibition of erythropoiesis [18,19,20], blood loss [21], iron or folate deficiency [22], hyperparathyroidism [23,24], marrow fibrosis, and aluminium excess [25,26], as well as a relative deficiency of EPO [8,9]. These will be discussed separately.

(i) Shortened red cell survival

Several studies have quantified red cell life-span by means of ^{51}chromium, DF^{32}P, or ^{14}C-cyanate labelling, and have shown this to be reduced in patients with chronic renal failure [16,17]. This is generally attributed to chronic low-grade haemolysis, which can decrease the life-span of the red cells from around 120 days to as little as 40-50 days. Nevertheless, this is not invariable, and many patients with end-stage renal disease have a normal red cell life-span.

The cause of the haemolysis is not known. Studies 30 years ago suggested that some intravascular substance or substances retained in patients with advanced renal failure shortened red cell survival; when red cells from such patients were infused into a normal subject, red cell life-span was restored to normal [27]. Neither haemodialysis nor peritoneal dialysis significantly improves red cell survival [28,29]. In the presence of normal kidneys, however, increased secretion of EPO would easily compensate for such a mild degree of haemolysis.

(i i) Inhibition of erythropoiesis

For more than 30 years, investigators have postulated that inhibitors of bone marrow activity play a significant causal role in renal anaemia. Four lines of evidence suggested the presence of erythropoietic inhibitors in patients with chronic renal failure: (1) *In vitro* erythropoiesis is impaired when uraemic serum is incubated with murine marrow cells in the presence of growth factors, including EPO [20]; (2) plasma levels of bioactive EPO in some anaemic haemodialysis patients are elevated [9]; (3) infusion of EPO-rich plasma from a patient with aplastic anaemia into several patients with advanced renal failure and anaemia failed to elicit a reticulocytosis [30]; and (4) a higher proportion of patients treated with continuous ambulatory peritoneal dialysis (CAPD) achieve normal haemoglobin levels than do haemodialysis patients [31]. Three substances have been incriminated as possible inhibiting solutes: parathyroid hormone [24], spermine [32], and ribonuclease [33]. Nevertheless, there are problems with accepting the arguments outlined above; these will be considered in turn.

(1) The *in vitro* studies, which have shown a reduction in EPO-induced proliferation of erythroid progenitor cells after addition of uraemic human serum, lack the controls required to support the selectivity and specificity of such inhibitors. Thus, the crude extract of the parathyroid gland was inhibitory not only to erythroid, but also to granulocyte progenitor cell lines [24]. In addition, purified PTH failed to inhibit *in vitro* erythropoiesis [34]. Spermine, likewise, inhibited both erythroid and granulocyte progenitor cell lines [35], and furthermore blood levels of spermine are not elevated in dialysis patients [36]. Ribonuclease-induced inhibition of erythropoiesis occurred only when pharmacological concentrations were added to the culture medium [33]. When mouse, rat, or dog erythroid marrow cells were used in culture, both *in vitro* granulopoiesis and megakaryocytopoiesis were inhibited, yet white cell and platelet counts in patients with chronic renal failure are usually normal [37]. Of more significance is that when an entirely autologous *in vitro* culture system was used, no inhibition of erythroid progenitor cells with uraemic sera was observed [38].

(2) Using bioassay results from concentrated serum, Caro et al. [9] found that some uraemic patients had serum EPO levels 3 to 4 times normal. Since these patients remained anaemic it was suggested that erythroid inhibitors suppressed the marrow response to the modest elevations of EPO. However, concomitant quantitation of erythropoiesis was not assessed, and hence an isolated elevated serum level is difficult to interpret. Furthermore, for the degree of anaemia reported, one would expect EPO levels of around 10 to 100 times normal in subjects with normal renal function [9]. Subsequent studies have also consistently failed to document significantly elevated serum EPO levels in uraemic anaemic patients [39].

(3) Although Essers et al. [30] found a reticulocytosis only in patients with mildly elevated blood urea and not in those with severe azotaemia after infusion of EPO-rich plasma, the number of subjects studied was small, and quantitative ferrokinetics were not performed. Eschbach et al. [40] later performed similar studies in uraemic anaemic sheep and obtained the same erythroid response to EPO-rich plasma in uraemic sheep as in normal sheep. Using this sheep model, they found no evidence of either *in vivo* or *in vitro* inhibition of erythropoiesis by uraemic toxins.

(4) The final observation that has been used to argue for the existence of uraemic suppression of erythropoiesis is that the haematocrit of patients starting CAPD often rises to normal [31,41]. It has been postulated that this is due to the removal of high molecular weight erythropoietic inhibitors which are not cleared by haemodialysis. Such mechanisms, however, do not explain why only about one-half of patients on CAPD have a significant rise in haematocrit, and why, of those that do, most are unable to sustain the rise for more than 18 months [41].

(iii) Blood loss

Significant blood loss occurs in as many as 25% of patients with chronic renal failure, thus contributing to their anaemia [21]. The main reason for this is an increased bleeding propensity caused by qualitative platelet defects which develop in azotaemic patients [42]. This leads to blood loss from the gastrointestinal tract, within the skin, and from other sites. In addition, haemodialysis patients can have significant losses of blood associated with the dialysis procedure. Platelet dysfunction prolongs the bleeding time and impairs platelet aggregation *in vitro* [43]. Several mechanisms for this have been proposed, including decreased platelet factor 3 activity, reduced platelet levels of thromboxane A_2, an increase in prostacyclin, and suboptimal Factor VIII: von Willebrand complex activity.

(i v) Iron or folate deficiency

Although not primarily a major cause of renal anaemia, deficiencies of iron or folate can certainly exacerbate this condition [22]. Iron deficiency may occur secondary to poor dietary intake, e.g. through loss of appetite, or to chronic blood loss as discussed above. Folate deficiency may also be dietary in origin, and there is also some folate loss through the dialyser. The importance of recognising these factors is that they are eminently reversible, and preclude the need for EPO.

(v) Hyperparathyroidism/marrow fibrosis

Hyperparathyroidism, especially osteitis fibrosa, can interfere with erythropoiesis, as evidenced by the improvement in renal anaemia in some dialysis patients after subtotal parathyroidectomy [23]. As discussed previously, there is controversy over whether high PTH levels *per se* inhibit erythropoiesis, but it was the degree of marrow fibrosis, as judged by bone biopsy, and not the elevated PTH levels which correlated best as a predictor of erythropoiesis after subtotal parathyroidectomy.

(v i) Aluminium excess

Aluminium overload, resulting from chronic use of aluminium-containing phosphate binders and/or aluminium-containing dialysate produces a non-iron deficient microcytic anaemia which can aggravate the anaemia of chronic renal failure [25,26]. Treatment of the aluminium excess with weekly injections of desferrioxamine as a chelating agent results in an increase in red cell volume within three months, and in eventual improvement in the anaemia [11].

(v i i) Relative EPO deficiency

Patients with non-renal causes of anaemia generally have greatly increased serum levels of EPO of the order of 10 to 100 times normal. There is an exponential relationship between haematocrit and serum EPO levels in these individuals, such that a linear decrement in haematocrit generally results in a disproportionate increase in

serum EPO. In contrast, patients with renal anaemia have levels of serum EPO which are within the normal range of 17-30 mU/ml, i.e. they are inappropriately low for the degree of anaemia [8,9]. This is particularly marked in anephric subjects. Thus, relative EPO deficiency is unquestionably the major contributory factor in the pathogenesis of renal anaemia. Since over 90% of circulating EPO is normally produced in the kidney, once renal disease supervenes maximum EPO secretion is presumably blunted. This occurs even when EPO production is stimulated by the hypoxia caused by anaemia and other forms of impaired oxygen delivery [44]. Thus, the logical approach to the treatment of renal anaemia was the use of erythropoietin replacement therapy.

2. Traditional treatments for renal anaemia

Until recently, the only options, other than renal transplantation, for ameliorating the anaemia of end-stage renal disease, were androgen therapy and blood transfusion. Androgens became popular in the 1970's, and improve renal anaemia either by stimulating the erythroid marrow directly or, more likely, by stimulating renal or liver EPO production [5,6,7]. Of the commercially available androgens, only two became popular, namely nandrolone decanoate and fluoxymesterone. In a 6-month crossover study with three other androgens, nandrolone worked the best [7]. Nandrolone decanoate was given weekly, 100-200 mg intramuscularly, for at least 6 months [5]. If the haemoglobin did not rise significantly, or if the requirement for transfusion persisted, a 6-month trial with oral fluoxymesterone, 10-30 mg/day, was considered. Unfortunately, virilisation, hepatic dysfunction, and other side-effects have limited the use of these agents, which tended to be less effective if the anaemia was severe [5,6,7].

Red cell transfusion is clearly the most rapid way of raising the haemoglobin concentration, and hence improving anaemic symptoms. However, not only are the effects transient, thereby necessitating repeated administration, but transfusions are associated with significant risks: (i) transmission of infectious agents such as hepatitis B or C, cytomegalovirus, and HIV. Even with proper screening to reduce this risk to a

minimum, a small number of transfused patients will still be exposed [45]. (ii) Sensitisation to histocompatibility antigens. Transfusion-induced anti-HLA antibodies are a major reason why some dialysis patients are unable to receive a kidney transplant, or have difficulty finding a suitable match. (iii) Suppression of endogenous EPO production. Repeated transfusion may suppress marrow activity by switching off what little endogenous EPO production there is. This, in turn, sets up a vicious circle whereby more frequent blood transfusion becomes necessary [46]. (iv) Iron overload is common in dialysis patients who have become transfusion-dependent. This occurs because the iron load resulting from the transfusion (200 mg of iron per unit of blood) can exceed the body's limited ability to eliminate iron through fixed gastrointestinal losses, menstruation, and losses related to surgical procedures. The excess iron is initially stored in the reticuloendothelial system, but severe iron overload leads to tissue deposition and cellular dysfunction, particularly of the heart, liver, and pancreas [47]. (v) Transfusion reactions. While it is rare for these to be life-threatening, mild immune reactions are not uncommon. Furthermore, some patients are notoriously difficult to cross-match, and finding suitable blood for transfusion is a real problem.

3. Development of recombinant human EPO

The story of erythropoietin began in the mid-19th century with an astute clinical observation by a French physician, Denis Jourdanet, who noted that the blood of his surgical patients in the highlands of Mexico was more viscous than normal and contained increased numbers of red cells [48]. This was attributed to the low atmospheric pressure, and was believed to be advantageous for survival at high altitudes. However, the association was felt to be merely fortuitous, and it was not until the end of the century that it was demonstrated that red cell production was stimulated in mountain climbers exposed to a few weeks of low atmospheric pressure. A humoral stimulator of erythropoiesis was first postulated in 1906 by Carnot and Deflandre [49], but proved elusive until the 1950's when several key studies were performed. In 1950, Reissmann showed that exposure of only one of a pair of parabiotic rats to hypoxia was

sufficient to stimulate erythropoiesis in both [50]. In 1953, Erslev confirmed this effect by transferring plasma from anaemic to non-anaemic rabbits [12], and four years later Jacobson et al. [13] concluded from studies of anephric rats that this elusive factor or hormone, called erythropoietin, was produced by the kidney. This provided a logical explanation why patients with chronic renal failure were anaemic.

The quantities of EPO isolated from biological fluids never permitted more than brief replacement studies in anephric mammals. Nevertheless, Eschbach and colleagues in 1984 demonstrated that the anaemia in uraemic sheep could be completely reversed by repeated infusions of EPO-rich plasma obtained from normal sheep rendered anaemic by phenylhydrazine and repeated phlebotomy [40]. Obtaining sufficient quantities of EPO for clinical trials in humans, however, was impossible until the development of genetic bioengineering techniques. The first major breakthrough came in 1977 when Miyake et al. [51] first isolated and purified a few micrograms of human erythropoietin from 2500 litres of urine obtained from patients with aplastic anaemia. Using recombinant DNA technology, oligonucleotide probes coding for portions of the EPO molecule were constructed, and used to screen a human genomic library. A fragment containing EPO-coding sequences was then isolated and used to screen a cDNA library which was constructed from poly-A^+ RNA isolated from human foetal liver [52,53]. Having isolated and cloned the gene for human EPO, it was then inserted into a suitable mammalian vector (Chinese hamster ovary cells). Expression of this gene in this system made possible for the first time, the large-scale synthesis of human EPO with an identical structure and immunoreactivity to the native hormone [54,55].

4. Biochemistry and physiology of erythropoietin

Erythropoietin is a glycoprotein hormone which is generated mainly by the kidneys [10,13], although up to 10% may be produced in the liver [56,57]. The site of synthesis in the kidney remains controversial, with *in situ* hybridisation localising mRNA to peritubular interstitial cells [58,59], or to tubular epithelial cells of the renal cortex [60]. In its physiologically active form, EPO is a 165-amino acid

monomeric protein with a molecular weight of 30,400 daltons [55] of which approximately 40% is carbohydrate [61]. The carbohydrate residues of EPO are not required for its biological activity or target cell specificity when measured *in vitro* [62] but, in common with other plasma glycoproteins, prevent its rapid removal from the circulation.

The stimulus for the secretion of EPO is insufficient delivery of oxygen to the tissues to meet metabolic demands [63,64]. The EPO, in turn, results in increased erythropoietic activity in the bone marrow, and the release of larger numbers of mature red blood cells into the circulation. This process is under negative feedback control [65]. Thus, hypoxia causes the production of EPO; EPO induces the proliferation of red blood cells by the bone marrow; the increased red cell mass improves oxygen transport and delivery, thereby alleviating tissue hypoxia and switching off EPO production. Hypoxia may be caused by various factors including decreased atmospheric oxygen tension (as occurs at high altitude), anaemia, ischaemia, increased metabolic rate, respiratory disease, and toxins affecting the oxygen affinity of haemoglobin.

EPO influences erythropoiesis at several stages by stimulating the proliferation and differentiation of erythroid precursors, including burst-forming units-erythroid (BFU-E), colony-forming units-erythroid (CFU-E), erythroblasts, and reticulocytes [66,67]. EPO also stimulates the release of reticulocytes from the bone marrow into the bloodstream where they mature into erythrocytes. At a cellular level, erythropoietin exerts its action by binding specifically to receptors on the erythroid progenitor cells in the bone marrow. Recently, Sawada et al. [68] purified the erythroid colony-forming cells from BFU-E generated from peripheral blood, and showed specific binding of ^{125}I-labelled EPO to these cells by a single receptor (550 receptors per cell). As these cells matured to erythroblasts, specific binding declined, and there were no receptors for EPO on mature red cells. Once bound to its receptor, EPO sets in motion a series of events which culminate in the production of the erythrocyte. The first measurable processes are an increase in intracellular calcium and glucose uptake, followed by α and β globin gene transcription at 6 hours, and an

increase in transferrin receptor expression. By 12 hours haemoglobin is being produced [69].

EPO synthesised by recombinant DNA technology has been found to be identical to and indistinguishable from endogenous human urinary EPO, possessing the same physicochemical, immunological, and physiological/pharmacological properties [54,55]. It has an activity of greater than 200,000 units/mg protein based on the 2nd International Reference preparation [70]. In view of its extreme hydrophobicity, current formulations of rHuEPO in solution contain a carrier protein (human serum albumin) or a mixture of amino acids to reduce adsorptive losses.

5.i Metabolic fate of erythropoietin

Remarkably little is known about the distribution, metabolism, and elimination in humans of either the endogenous hormone or the recombinant product [71]. The volume of distribution of recombinant EPO is equivalent to, or slightly greater than, plasma volume. This suggests that EPO is largely distributed intravascularly, with any extravascular component being of minor importance. In a number of animal investigations, however, a more substantial extravascular distribution space has been found [72,73,74,75,76].

5.ii Metabolism and excretion: the role of the kidney

At present there are major difficulties in assessing the role of the kidney in the metabolism of EPO. There is only limited information in humans, and animal investigations with homologous hormone have utilised impure preparations and relatively insensitive assays. The metabolism of recombinant human EPO in experimental animals has been studied using a specific radioimmunoassay but the biological relevance of information derived from the use of a xenogeneic hormone is uncertain.

In the only human study of rHuEPO pharmacokinetics in individuals with varying renal function, Kindler et al. [77] provided evidence against a role for the kidneys in its

metabolism. Plasma half-lives of 8.5±1.0, 8.8±0.9, and 10.4±2.4 hours were obtained from patients with normal renal function (creatinine clearance >80 ml/min), moderately impaired renal function (creatinine clearance 10-50 ml/min), and end-stage renal failure (creatinine clearance <3 ml/min), respectively. Likewise, there was no change in the total body clearance between the 3 groups (0.1197±0.0005, 0.0855±0.0069, and 0.0803±0.0190 ml/min/kg respectively). Similarly, in two groups of normal and uraemic (5/6 nephrectomised) rats, Scigalla et al. [78] found no difference in either the plasma disappearance half-lives (12.8 vs 11.5 hours) or the renal clearances (0.04 vs 0.05 ml/min/kg) of biologically active ^{35}S-labelled rHuEPO, again suggesting that the kidneys were not primarily involved in the metabolism of EPO. Finally, Mladenovic and colleagues [79] reported that the half-life of infused homologous EPO-rich plasma in both normal and uraemic sheep was approximately 9 hours, and was independent of renal function, the plasma clearances being 3.7±2.0 and 3.3±1.8 ml/min, respectively.

In contrast, there are a number of animal studies which do suggest a role for the kidney in the metabolism of EPO [72,76,80,81,82]. Spivak and Hogans [82] recently found that there was progressive accumulation of iodinated rHuEPO in the kidneys of anaesthetised rats compared with other organs, and concluded that the kidney was involved to a small extent in the catabolism of the hormone. Furthermore, this was supported by their studies with desialated, oxidised EPO, which also appeared to accumulate in the kidneys as opposed to other organs. In a pharmacokinetic study in healthy and anephric dogs, Fu et al. [76] found a slower clearance of ^{125}I-labelled recombinant human EPO in the anephric animals (half-life = 13.8±1.4 hours; Cl = 0.008±0.001 l/kg/hour) compared with the intact dogs (half-life = 9.0±0.6 hours; Cl = 0.011±0.001 l/kg/hour). Emmanouel et al. [72] compared the metabolic clearance rates of ^{125}I-labelled purified human EPO in control and acutely nephrectomised rats, and found a 32% reduction after nephrectomy (0.19±0.03 vs 0.13±0.01 ml/min/kg). This was considerably larger than could be explained solely by cessation of urinary excretion of the hormone (7.4%), and thus a role for the kidneys in the degradation of

EPO was suggested by the authors [72]. Two other groups of workers [80,81] have found significantly prolonged plasma disappearance half-lives of endogenous EPO in rats after bilateral nephrectomy, compared with controls.

It is well-recognised that a small proportion of EPO is excreted unchanged in the urine [51]. This, however, amounts to no more than a few nanograms of protein over a 24-hour period. Thus, in both animal and human studies, urinary excretion of EPO contributes only 3-10% of its overall elimination [72,73,77,83]. Recently, Kindler et al. [77] found that the renal clearance of rHuEPO in humans accounted for less than 3% of total body clearance, and was independent of renal function. Renal clearance rates of endogenous EPO ranging from 0.06 to 0.67 ml/min were found in patients with non-renal anaemia due to either leukaemia, malignancy, or marrow aplasia [83]. This represented a urinary excretion rate of about 10% of the total daily loss. In rats, 7.4±0.8% of the total body clearance of ^{125}I-labelled purified human EPO could be accounted for by excretion of the labelled hormone in the urine [72]. Urinary clearance of ^{125}I-EPO amounted to <0.3% of the glomerular filtration rate, and there was no detectable arteriovenous concentration difference of ^{125}I-EPO across the kidney. Weintraub et al. [73] found that between 4% and 7% of purified sheep EPO disappearing from plasma was recoverable in the urine of unanaesthetised dogs. Thus, it would appear that peripheral EPO disposal occurs mainly via non-renal mechanisms. The liver, and possibly the erythropoietic tissue of the bone marrow, may be implicated in this context.

5.iii Metabolism: the role of the liver

In experimental models, some workers have suggested hepatic degradation of EPO [84,85], while other groups were unable to confirm that intact EPO was catabolised in the liver [81,86,87,88]. Most of these discrepancies can be attributed to the previous lack of standardised assay methods for measuring plasma EPO levels. In isolated perfused dog livers, Roh et al. [85] demonstrated rapid hepatic degradation of homologous EPO. Both Fischer et al. [86] and Kukral et al. [87], however, found no

evidence of EPO catabolism in the liver. A more recent study investigating the pharmacokinetics of rHuEPO degradation in the isolated perfused rat liver also demonstrated no change in either immunoreactive rHuEPO or tracer radioactivity of ^{35}S-cysteine rHuEPO in the perfusate [88]. Only minute amounts of immunoreactive EPO, and on average 0.37% of total tracer added, were retrieved in the bile collected [88]. In rats treated with the hepatotoxic agent d-Galactosamine-HCl a small but significant prolongation of the plasma disappearance half-life of endogenous EPO was found compared with control rats (164 and 105 minutes, respectively) [81]. This, however, was minor compared with the effect of bilateral nephrectomy, which increased the half-life to 266 minutes. The combination of nephrectomy and d-Galactosamine-HCl treatment did not result in a significant further prolongation of the half-life [81]. In experiments using isolated perfused rat livers, the same workers found no change in the perfusate EPO titre during 4 hours of perfusion, and the results of both these studies led the authors to suggest that hepatic degradation of EPO in rats was only minimal compared with the action of the kidney.

Thus, as with the studies investigating the role of the kidney in EPO catabolism, there is controversy regarding the contribution of the liver to the metabolism of EPO, and as yet there is no information in humans.

5.iv Metabolism: the role of the carbohydrate moiety of EPO

As discussed earlier, EPO is a heavily glycosylated protein with 40% of its molecular size accounted for by carbohydrates [61]. The carbohydrate residues of EPO are not required for its biological activity or binding to target cells *in vitro* [62], but they have a crucial role in maintaining the stability of EPO in circulation. If the terminal sialic acid groups are removed from the EPO molecule, the resultant asialoerythropoietin is rapidly cleared from the circulation by hepatic cells. This, in turn, causes apparent loss of biological activity *in vivo.* Fukuda et al. [89] obtained a half-life of about 2 hours for ^{125}I-rHuEPO in rats. In contrast, the desialated EPO was cleared from the circulation within 10 minutes. It was suggested that the galactose

binding protein of hepatic cells is involved in this process [89]. Simultaneous work by Spivak and Hogans [82] yielded similar results with 96% of the desialated ^{125}I-rHuEPO being cleared from the plasma with a half-life of 2 minutes, in contrast to 180 minutes for the intact hormone. The bulk of the desialated EPO accumulated in the liver where it was rapidly catabolised and its breakdown products released back into the plasma. Interestingly, in contrast to the unmodified EPO, there was also early accumulation of desialated hormone in the kidneys, marrow, and spleen. Oxidation of desialated EPO restored its plasma recovery and clearance to normal, but rendered it biologically inactive [82].

6. Early clinical experience with recombinant human erythropoietin

Clinical trials of the recombinant product began in Seattle and London/Oxford in 1986, and its efficacy in ameliorating the anaemia in patients with end-stage renal failure was established at an early stage. Winearls et al. [90] observed an increase in the mean haemoglobin concentration from 6.1 to 10.3 g/dl in 10 haemodialysis patients over an 11-week period. The need for regular blood transfusion was abolished. Nine of the patients reported an improved sense of well-being, while eight noted an increase in exercise tolerance. Eschbach and co-workers [91] treated 25 haemodialysis patients with doses of EPO ranging from 1.5 to 500 U/kg intravenously thrice weekly. They observed a dose-dependent rise in the haematocrit with doses of 15 U/kg or higher. This favourable haematological response to EPO was subsequently confirmed by Casati et al. [92] who reported a mean haemoglobin rise from 6.2 to 10.5 g/dl in 14 patients, and Bommer et al. [93] who observed a median haematocrit increase from 19.4% to 30.0% or 32.5% depending on the dose used. These studies also documented significant improvements in patient well-being, physical performance, appetite, libido, and symptoms of Raynaud's phenomenon, as well as a reduction in headaches and fatigue. Several patients were able to return to work as a result of treatment with EPO. No antibodies against EPO were detected.

A number of side-effects were noted in these early clinical trials. These included 'flu'-like symptoms, chills, and transient bone pain, but of greater concern was the development of hypertension (occasionally severe, resulting in encephalopathy), and thrombosis of the arteriovenous fistula in 7 out of the 62 patients included in the initial 4 clinical trials [90,91,92,93]. One cerebral thrombotic lesion was also reported [92]. All 4 studies noted an increased tendency for clotting in the dialysis lines, requiring an average 20% increase in the dose of heparin used during dialysis.

9. AIMS OF STUDY

At the time of commencing this work (in April 1988), the efficacy of intravenous EPO in ameliorating renal anaemia in haemodialysis patients had been established (see previous section). The optimal dosage regimen and route of administration, however, had not been ascertained, no studies had been conducted in CAPD patients, and little was known about the secondary effects of increasing the haematocrit with EPO.

The aim of this project was, firstly, to establish the pharmacokinetics of EPO using different routes of administration, with a view to rationalising the most appropriate dosage regimen. Secondly, the dose-effect relationship of the drug on the haematological response was examined in patients with renal anaemia, comparing different doses and routes of administration. Thirdly, the secondary beneficial effects of EPO were studied in terms of long-term cardiorespiratory function, as well as recording any adverse effects. In relation to the latter, the effects of EPO on various haemorheological and coagulation parameters were monitored, along with assessing arteriovenous fistula blood flow during treatment. In addition, several studies using radiolabelled EPO were carried out to elucidate the absorption kinetics and metabolic fate of this hormone. Finally, based on the experience gained during this project, a series of practical guidelines and a clinical algorithm for the use of EPO in treating renal anaemia were compiled.

CHAPTER 1

PHARMACOKINETICS OF RECOMBINANT HUMAN ERYTHROPOIETIN

INTRODUCTION

In common with insulin and other therapeutic protein hormones, erythropoietin is inactivated in the stomach and therefore must be administered parenterally. The early clinical trials in haemodialysis patients used intravenously-administered EPO, but there was no information at that time as to what was the optimum dosage regimen and route of administration for this new drug [94]. Furthermore, the intravenous route was clearly impractical for long-term use in patients on continuous ambulatory peritoneal dialysis (CAPD) who lacked ready vascular access. A study in rabbits, however, had recently shown that 60% of the EPO added to peritoneal dialysis fluid, and 98% of EPO injected into an empty peritoneal cavity, was absorbed [95]. This suggested that intraperitoneal administration of EPO might be a practical therapeutic option for CAPD patients.

The purpose of the study described in this chapter was to determine and compare the single-dose pharmacokinetics of EPO administered intravenously (IV), intraperitoneally (IP), and subcutaneously (SC) to a group of stable CAPD patients.

PATIENTS AND METHODS

Patients

Eight patients on regular CAPD were studied. They consisted of 4 males and 4 females, with a mean age of 62 years (range 43-76 years), and a mean weight of 67 kg (range 52-85 kg). The aetiology of their renal disease was varied, and included chronic glomerulonephritis, hypertensive nephropathy, diabetic nephropathy, polycystic kidneys, and benign nephrosclerosis. Patients had been on CAPD for a range of 7 to 70 months (median 17 months) and their mean haemoglobin concentration at the start of the study was 10.5 g/dl (range 8.6-13.9 g/dl). All were well at the time of the study and none had experienced any episodes of peritonitis within the previous 6 months.

Protocol

Patients were admitted to hospital for the study. Baseline serum levels of EPO ranged from 18 to 46 mU/ml, with the exception of the patient with polycystic kidneys who had a concentration of 236 mU/ml. Each patient received EPO (Boehringer Mannheim GmbH, Germany) on 3 occasions separated by at least 4 weeks. The doses given were IV 120 U/kg (mean dose per patient = 8043 U), IP 50,000 U, and SC 120 U/kg. Prior to administration of the drug, three baseline blood samples were taken over a 1-hour period for measurement of serum EPO; thereafter samples were withdrawn at 15 mins, 30 mins, 1, 2, 3, 4, 5, 6, 7, 8, 12, 18, and 24 hours. Additional samples were taken at 32, 48, 72, and 96 hours during the subcutaneous phase of the study. Serum was separated in a refrigerated centrifuge and stored at -20^{o}C before assay in a single batch.

The dialysis regimen was kept constant throughout the initial study period with 3 bags of solution containing 1.36% glucose administered over the first 24 hours using 8-hour, 4-hour, and 12-hour (overnight) dwells sequentially. Dialysate samples were taken from each of the 3 bags at the end of their dwell. EPO levels were measured by radioimmunoassay using a specific polyclonal antibody [96]. In brief, 100 µl of serum

or standard (2nd International Reference preparation of human urinary EPO; 10-500 mU/ml) and 100 µl of diluted EPO anti-serum were incubated for 24 hours before addition of ^{125}I-radiolabelled EPO. Separation of bound versus free ligand was accomplished by use of a second antibody technique. The interassay coefficient of variation was <8% over a range of EPO concentrations.

Losses of EPO in the dialysate were calculated by measuring the total amount of EPO recovered from the peritoneal dialysis effluent during the first 24 hours and expressing this as a percentage of the total dose given.

Data analysis

The decline in individual serum EPO concentrations was mono-exponential, and results were therefore analysed according to the equation $C = Ae^{-kt}$ (C = serum concentration at time t; k = elimination rate constant of a single-compartment open model; A = intercept with the ordinate) after baseline levels were deducted. This analysis was performed on a microcomputer by standard curve stripping procedures.

Areas under the curves (AUC) were determined by the trapezoidal method with extrapolation to infinity. Serum clearance (Cl) was calculated from the dose divided by the AUC; distribution volume (Vd) was calculated as described by Ritschel [97].

Bioavailability (f) of IP and SC EPO was assessed using the formula,

$$f = \frac{AUC_{IP\ (or\ SC)} \ / \ Dose_{IP\ (or\ SC)}}{AUC_{IV} \ / \ Dose_{IV}}$$

RESULTS

IV administration of EPO 120 U/kg

Analysis of the serum decay curve indicated that EPO was distributed in a single compartment during the first 24 hours with a mono-exponential decay (Fig. 1). Serum levels peaked at 3959±758 (SD) mU/ml 15 minutes after administration, and fell to 558±181 mU/ml at 24 hours. The mean serum elimination half-life of EPO was 8.2 hours (range 6.2-10.2 hours) with an elimination rate constant (k_{el}) of 0.087/hr. The AUC was 38302±9375 mU/ml/hr to 24 hours and 45102±11405 mU/ml/hr when extrapolated to infinity. The mean apparent volume of distribution was 0.033 l/kg (range 0.021-0.063) and the mean serum clearance rate was 0.047 ml/min/kg (range 0.032-0.085).

Losses of EPO in the peritoneal dialysis fluid ranged from 1.7 to 3.0% (mean 2.3%) of the total IV dose over the first 24 hours.

IP administration of EPO 50,000 U

Serum EPO levels became detectable within 2 hours of commencement of the dwell, reaching a peak of 375±123 mU/ml at 12 hours, and falling slowly to 287±135 mU/ml at 24 hours with a k_{el} of 0.032/hr (Fig. 2). The mean AUC to 24 hours was 6432±2150 mU/ml/hr giving a bioavailability for IP EPO of only 2.9% (range 1.2-6.8%) (Fig. 3).

SC administration of EPO 120 U/kg

There was a significant rise in the serum levels of EPO 2 hours after administration, and a peak concentration of 176±75 mU/ml was attained at 18 hours (Fig. 4). EPO levels then decayed exponentially to 49±26 mU/ml at 96 hours with a k_{el} of 0.025/hr. The mean AUC to infinity was 9610±4862 mU/ml/hr, indicating a bioavailability of 21.5% (range 11.3-36.0%) (Fig. 3).

Simulated multiple-dose pharmacokinetic profile

Using the method quoted by Ritschel [98], elimination and absorption rate constants were calculated for the SC phase of the study. These were estimated as 0.0163/hr and 0.1684/hr respectively. The constants were then used to predict the steady state serum concentrations of EPO at different dosage intervals (Table 1).

DISCUSSION

This was the first comprehensive pharmacokinetic study of EPO in CAPD patients to be reported [99]. The half-life of IV EPO in CAPD patients was similar to that previously found in haemodialysis subjects [77,100,101] suggesting that peritoneal dialysis itself has no significant effect on EPO clearance. The trivial amounts of EPO lost in the dialysate, which for practical purposes can be discounted, is further evidence in support of this statement. Cumulative results from 15 pharmacokinetic studies of EPO involving 141 patients were reviewed recently [71], and the mean plasma elimination half-life in this series ranged from 4.0 to 11.2 hours. The subjects studied included a mixture of normal healthy volunteers, patients with varying degrees of renal impairment not yet requiring dialysis, and haemodialysis and CAPD patients. The current evidence suggests that the half-life and clearance of EPO is independent of renal function [71,77].

The volume of distribution in this study was of the same order as estimated plasma volume and similar to that found in two recent studies in haemodialysis patients [100,101], although Kindler et al. [77] in a separate haemodialysis study reported a distribution volume of 1.5-2.0 times plasma volume. Animal studies have shown a similar variability in distribution volume, from being equal to plasma volume in sheep [79] and rats [102], to being 2-3 times plasma volume in dogs [73,76] and rats [74]. All the older animal studies, however, used impure preparations and highly variable bioassays, which may partly explain these apparent discrepancies.

A study on EPO absorption from the peritoneal cavity in rabbits [95] demonstrated that 60% of EPO in dialysate, and 98% of EPO injected directly into an empty peritoneal cavity, was absorbed. This suggested that IP administration of EPO to CAPD patients might be a suitable route. The bioavailability data from the present study, however, indicate that in order to achieve the same serum profile the IP dose needs to be 5- to 10-fold higher than that administered SC. Thus, even though EPO may be effective given IP [103], this route of administration results in an unacceptable wastage of a costly drug. A number of other studies have subsequently confirmed the low bioavailability of IP-administered EPO which was reported as 2.5% (4-hour dwell) and 3.6% (12-hour dwell) in a study by Boelaert et al. [104], 6.8% in a study by Kampf et al. [105], and 8.5% in a study by Gahl et al. [106].

There are two further points of interest arising from the IP serum curve in the present study. Dialysis fluid was drained out from the peritoneal cavity after the initial 8-hour dwell and replaced with a fresh bag containing no EPO. Despite this, serum EPO levels continued to rise by a further 50 mU/ml to peak at 12 hours. Also, the subsequent elimination of EPO from the serum (k_{el} = 0.03/hr) occurred much more slowly than was evident after IV dosing (k_{el} = 0.09/hr). Both these findings suggest that there was continued absorption of EPO into the bloodstream for several hours after the peritoneal cavity was flushed, possibly due to pooling within the lymphatic system.

The bioavailability of EPO given subcutaneously was seven-fold higher than that of intraperitoneal EPO, but was still only 22%. Subsequent studies have reported estimates of bioavailability after SC administration ranging from 10.2% [104] to 49% [105], although most quote values of around 20% [107,108,109]. This is considerably lower than that found previously for subcutaneous insulin (50-80%) [110], heparin (40%) [111], and growth hormone (71%) [112]. There are two possible explanations for this. Firstly, EPO may be degraded by peptidases in the skin as has been demonstrated for insulin [113], or secondly the larger size of the EPO molecule (molecular weight 30,400 daltons) may impede absorption. Emanuele and Fareed [111] recently showed a striking negative correlation between the size and

bioavailability of five different molecular weight heparins administered subcutaneously. The bioavailability ranged from 3% to 93% depending on the molecular weight of the heparin fraction tested.

It has been reported that haemodialysis patients treated with IV EPO will maintain their haemoglobin concentration when switched to a lower dose of EPO administered subcutaneously [114]. This suggests that the extremely high peaks obtained after IV EPO are not required for its therapeutic efficacy. For this reason, and since EPO levels after SC administration are maintained for a longer period (3-4 days), it would seem reasonable to recommend the subcutaneous route for treating both haemodialysis and CAPD patients with EPO.

From the simulated multiple-dose pharmacokinetic data, whilst accepting the limitations of extrapolating multiple-dose from single-dose pharmacokinetics, a sensible starting dose appeared to be 60-120 U/kg twice weekly, with adjustments in the light of the haemoglobin response. This dose was subsequently used for treating two groups of anaemic dialysis patients, and the results are presented in the next chapter of this thesis. Several other groups of workers have also used SC-administered EPO as the basis of their treatment regimens, with encouraging results [115,116,117,118,119]. The bioavailability of SC EPO, however, is still less than ideal, a feature likely to be related to its molecular size. Assuming, however, that the active moiety of the EPO molecule is but a fraction of its entire structure, this raises the possibility of synthesising a smaller molecule of similar efficacy but with greater bioavailability. In addition, further work is required to investigate the relationship between EPO pharmacokinetics and pharmacodynamics, to determine the "ideal" plasma concentrations and time profiles of EPO, and to establish the optimal duration of interaction of EPO with its receptor to induce the maximum therapeutic effect.

Table 1 Predicted steady state serum EPO levels at different dosage intervals following multiple dosing with subcutaneous EPO 120 U/kg.

	DOSAGE INTERVAL (hr)		
	48	72	96
t_{max}	11.3	12.9	13.8
$C_{ss\ max}$	377	281	242
$C_{ss\ min}$	224	119	70

t_{max} = time to reach maximum concentration at steady state (hr)

$C_{ss\ max(min)}$ = maximum (minimum) serum concentration at steady state (mU/ml).

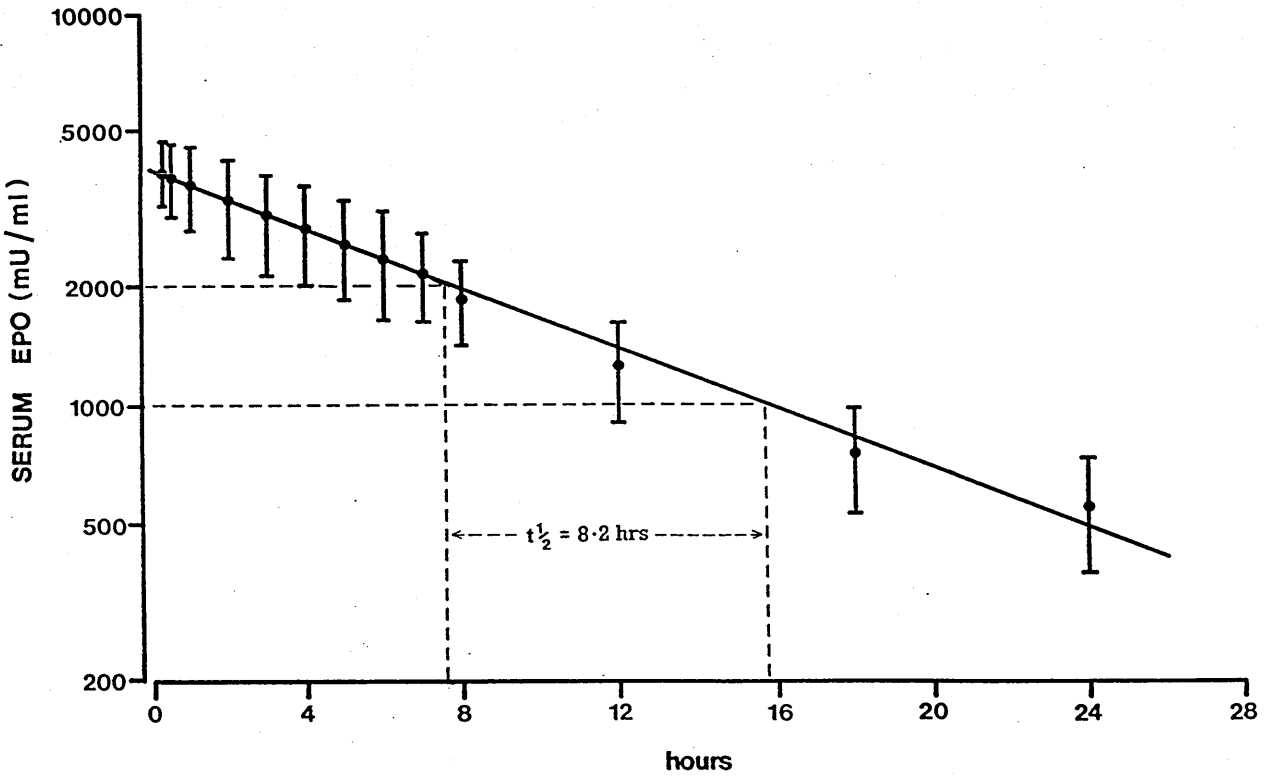


Fig. 1 Serum EPO profile following IV administration of 120 U/kg in 8 stable CAPD patients.

Results expressed as means ± SD.

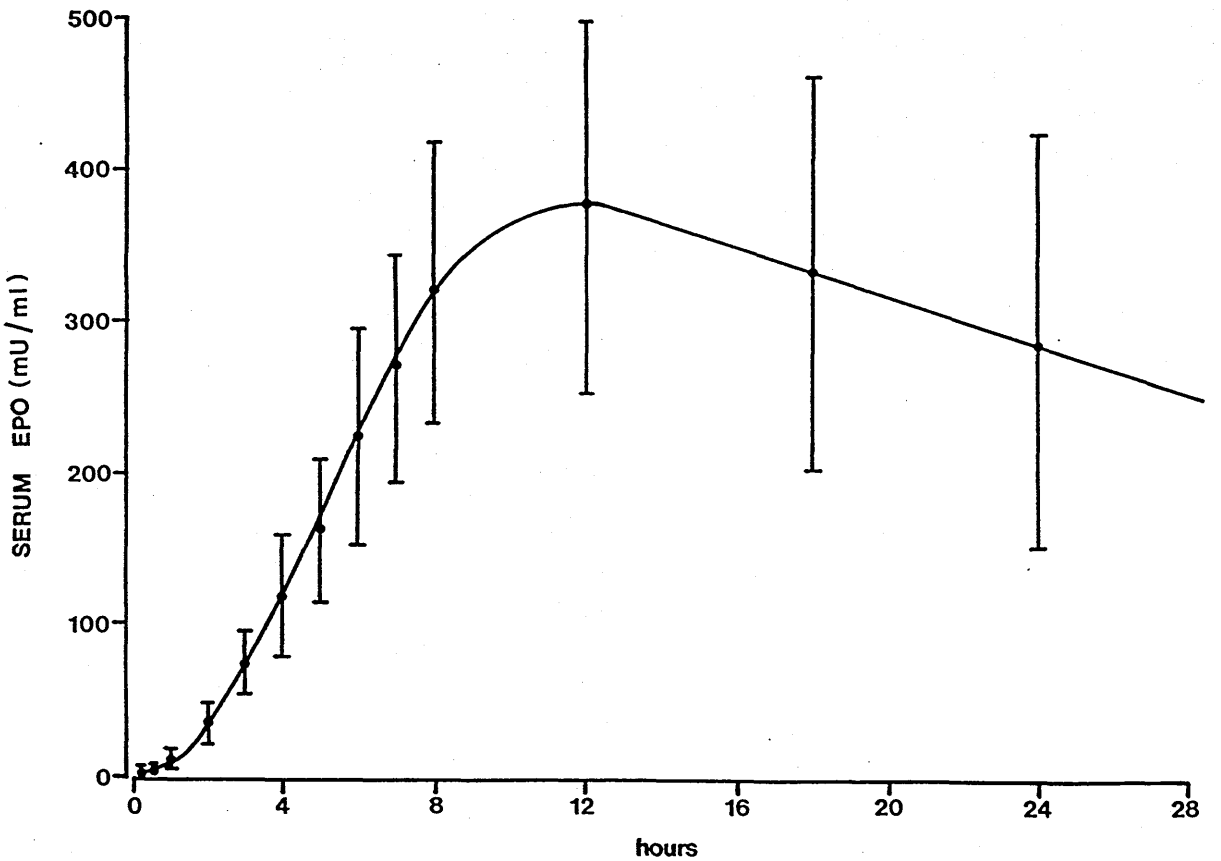


Fig. 2 Serum EPO profile following IP administration of 50,000 units in 8 stable CAPD patients.

Results expressed as means ± SD.

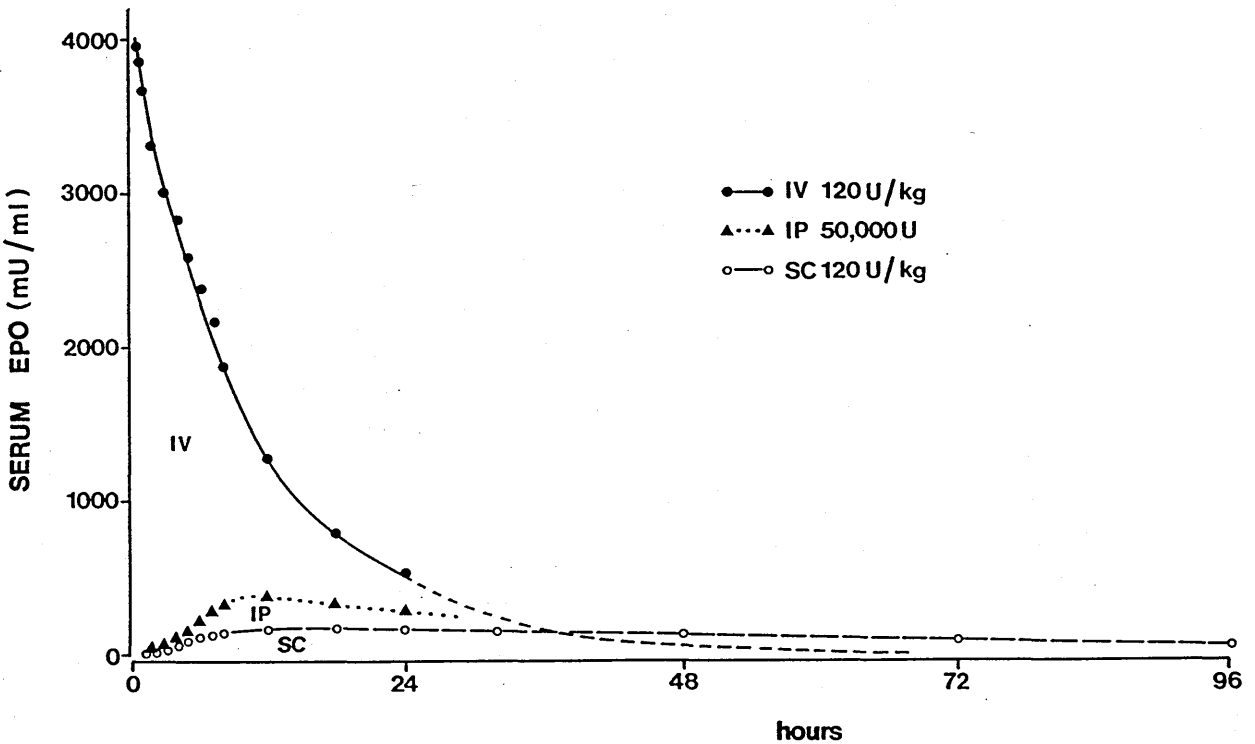


Fig. 3 Bioavailability of EPO following IP (50,000 U) and SC (120 U/kg) administration in 8 stable CAPD patients relative to an IV dose of 120 U/kg.

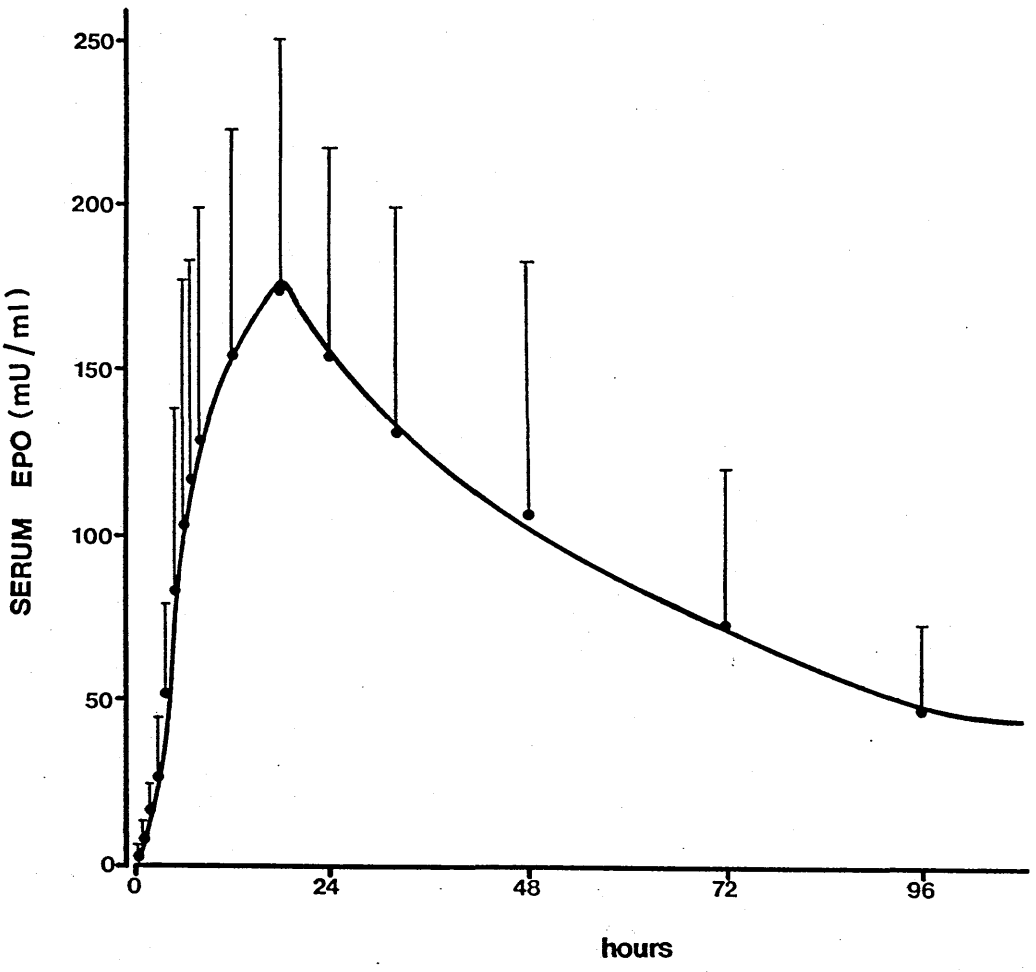


Fig. 4 Serum EPO profile following SC administration of 120 U/kg in 8 stable CAPD patients.

Results expressed as means ± SD.

CHAPTER 2

HAEMATOLOGICAL RESPONSE TO RECOMBINANT HUMAN ERYTHROPOIETIN IN PATIENTS WITH RENAL ANAEMIA

INTRODUCTION

At the time of starting this project, the efficacy of EPO in being able to correct the anaemia of end-stage renal disease in haemodialysis patients had recently been established [90,91,92,93]. Such studies had employed EPO given by the intravenous route twice or thrice weekly at the end of dialysis. The pharmacokinetic investigation detailed in the previous chapter indicated that the subcutaneous route might be preferable not only for CAPD patients who lack ready vascular access, but also for haemodialysis subjects. The purpose of the study described in this chapter was to examine the haematological response to EPO in 3 groups of dialysis patients (2 haemodialysis, 1 CAPD) using both IV and SC routes of administration and 2 different dosage regimens. To this end, the haemoglobin concentration, reticulocyte count, red cell indices, white cell and platelet counts, and parameters of iron status were monitored regularly over the first few months of EPO therapy. In addition, the effect of EPO on a number of ferrokinetic and blood volume measurements were studied in the CAPD patient group.

PATIENTS AND METHODS

Patients

Three groups of dialysis patients were included in this study:-

Group 1 : 10 haemodialysis patients treated with IV EPO 240 U/kg/wk

Group 2 : 15 CAPD patients treated with SC EPO 120 U/kg/wk

Group 3 : 13 haemodialysis patients treated with SC EPO 120 U/kg/wk

Further details of these patients with regard to age, sex, weight, aetiology of renal failure, duration on dialysis, and previous transplantation and transfusion history are shown in Tables 2, 3, and 4. As might be expected, the CAPD patients, on average, were older, and by chance appeared to include relatively more females. The other parameters, however, were similar, and the mean starting haemoglobin concentrations were likewise fairly comparable among the three different patient groups (6.3±0.6 g/dl for Group 1; 6.2±0.8 g/dl for Group 2; and 6.9±0.7 g/dl for Group 3). All patients had

normal serum ferritin, vitamin B_{12}, and folate levels, and no obvious cause of anaemia other than their renal failure.

Group 1 patients were given 120 U/kg of EPO (Boehringer Mannheim GmbH, Germany) intravenously twice weekly at the end of dialysis. Patients in groups 2 and 3 received a subcutaneous injection of EPO (Boehringer Mannheim GmbH), 60 U/kg, into the skin of the upper arm (mid-deltoid region) twice weekly. Subsequent dosages were titrated according to the initial response: a rise in haemoglobin concentration of less than 1 g/dl/month led to a 50%, and occasionally a 100%, increase in the dose of EPO. Similar decrements in dosage were instituted if the haemoglobin concentration rose too rapidly and when the target haemoglobin level of 10-12 g/dl had been achieved.

All patients received oral iron supplementation in the form of ferrous sulphate 200-600 mg or ferrous gluconate 300-600 mg per day. Intravenous iron was also given as required if there was biochemical evidence of functional iron deficiency (transferrin saturation <20%).

Methods

Full blood count indices including haemoglobin concentration, haematocrit, mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), and white cell and platelet counts were measured weekly on a Technicon (Basingstoke, UK) H1 automated blood cell analyser during the first 4 months of EPO therapy, and measured monthly thereafter. Reticulocyte counts were estimated from a blood film stained with brilliant cresyl blue by counting the number of reticulocytes in 500 red cells. This was carried out weekly over the first 4 months of treatment by a single observer. Serum ferritin was measured every 2 weeks during the first 4 months of therapy using a sensitive immunoradiometric assay [120], and serum iron and total iron binding capacity (TIBC) were determined using a standard method [121] at the same time intervals. After 4 months of EPO, these parameters of iron status were measured monthly. Percentage transferrin saturation was expressed as the ratio of serum iron to serum TIBC.

A number of biochemical parameters were also monitored regularly throughout EPO treatment; these included standard urea and electrolyte measurements, liver function tests (serum albumin, protein, bilirubin, alkaline phosphatase, gamma glutamyl transferase, aspartate transaminase, and uric acid levels), and calcium and phosphate levels. All of these were measured on automated biochemical analysers.

Twelve of the CAPD patients underwent detailed ferrokinetic and blood volume studies prior to EPO, and eight of these had repeat measurements performed after achieving their target haemoglobin concentration of >10 g/dl. These studies were carried out using the method of Cavill & Ricketts [122] in which the patient's own transferrin was labelled with ^{59}Fe. After removal of unbound ^{59}Fe, the labelled transferrin was re-injected into the patient. The time course of the clearance of ^{59}Fe from the plasma and its reappearance in circulating red cells was monitored over the subsequent 10-14 days. This allowed the quantitation of total erythropoiesis (marrow iron turnover), effective erythropoiesis (red cell iron turnover), and ineffective erythropoiesis, as well as measurement of non-erythroid iron turnover and mean red cell life-span. In addition, the circulating red cell volume was determined using ^{51}Cr-labelled autologous red cells, and plasma volume by dilution of the ^{59}Fe-labelled transferrin. Whole blood volume was calculated from the sum of these two latter parameters [122].

Statistics

Analysis of variance and the two-tailed paired t test were used to assess the statistical significance of any changes during EPO therapy. Results are expressed throughout as means ± standard deviation.

RESULTS

(i) Haemoglobin concentration

This rose in 9 of the Group 1 patients from a mean value of 6.4±0.6 g/dl before EPO, to 8.6±1.2 g/dl after 4 weeks, 10.3±1.7 g/dl after 8 weeks, 11.0±0.9 after 12 weeks, and 11.5±0.7 g/dl after 16 weeks (Fig. 5). There was, however, considerable variation between patients in the rate of response to EPO over this period despite receiving the same dose of drug (Fig. 6). With further adjustments in the dose of EPO, the mean haemoglobin concentration was kept within the target range of 10-12 g/dl for up to 12 months of treatment (10.8±0.7 g/dl).

One patient (SW) showed a poor response to EPO over the first few months of treatment. Further investigation revealed that she had moderate marrow fibrosis on trephine biopsy and intermittent gastrointestinal blood loss from gastric erosions (Fig. 7). Nevertheless, she eventually attained her target haemoglobin level after nearly 2 years of therapy and following a switch to daily subcutaneous administration.

Ten of the 15 CAPD patients in Group 2 demonstrated a complete response to EPO (attainment of target Hb >10 g/dl) (Fig. 8). Four patients showed a partial response (significant rise in haemoglobin concentration but never reaching their target), and one patient (MF) exhibited no response at all (Fig. 8). Possible reasons for the incomplete response included chronic infection in two patients, and marrow fibrosis (due to secondary hyperparathyroidism) in one. The final patient died suddenly at home after 13 weeks of therapy before she had a chance to reach her target. The "non-responder" (MF) was subsequently found to have liver metastases from a breast carcinoma diagnosed two years previously, but was believed to be disease-free at the time of starting EPO. The mean (±SD) haemoglobin concentration in the ten "responders" was 6.5±0.9 g/dl before EPO, 7.9±1.4 g/dl at 4 weeks, 8.8±1.4 g/dl at 8 weeks, 10.0±1.5 g/dl at 12 weeks, 10.1±1.7 g/dl at 16 weeks, 11.4±1.3 g/dl at 8 months, and 11.3±1.2 g/dl at 12 months.

Ten of the 13 haemodialysis patients in Group 3 showed a complete response to EPO (haemoglobin 6.8±0.7 g/dl before treatment, 8.4±0.9 g/dl at 4 weeks, 9.6±1.7

g/dl at 8 weeks, 10.3±1.8 g/dl at 12 weeks, 10.0±1.4 g/dl at 16 weeks, 10.9±1.7 g/dl at 8 months, and 11.1±1.3 g/dl at 12 months). The remaining three patients all received renal transplants before reaching their target haemoglobin level.

A comparison of the mean haemoglobin concentrations over the first 12 months of treatment revealed very similar results for the 3 groups of "responding" patients (Fig. 9). This occurred despite the intravenously-treated patients (Group 1) receiving twice the starting dose of EPO given to the patients treated subcutaneously (Groups 2 and 3).

Several factors were noted to influence the response to EPO. The commonest of these was functional iron deficiency, and many patients required intravenous iron supplementation as discussed later. An example of this is shown in Fig. 10. Infection, both acute and chronic, also appeared to inhibit the response to EPO, as is illustrated in Fig. 11. This shows the dramatic fall in haemoglobin concentration in a 52-year-old man who developed a severe chest infection, having been stable at his target haemoglobin level for over a year. This did not respond to a doubling in the dose of EPO, and it was only after resolution of his infection that his haemoglobin concentration began to climb towards its previous value.

(ii) Maintenance doses of EPO

EPO dosages were adjusted according to the haemoglobin response, the aim being to maintain the haemoglobin concentration within the range of 10-12 g/dl. The maintenance doses of EPO required to do this after 12 months of treatment varied considerably, from 85 to 720 U/kg/wk in Group 1 patients, 60 to 360 U/kg/wk in Group 2 patients, and 60 to 240 U/kg/wk in Group 3 patients (Fig. 12). Despite this variability, the maintenance doses of the subcutaneously-treated patients (medians 120 and 123 U/kg/wk) were generally lower than for the intravenously-treated subjects (median 188 U/kg/wk).

(i i i) Reticulocyte counts

The mean absolute reticulocyte count for the Group 1 patients rose significantly after 1 week of EPO treatment from 30±15 $x10^9$/l to 110±61 $x10^9$/l, and was maintained at this level over the first 6 weeks of therapy (123±50 $x10^9$/l). Thereafter, it appeared to settle to lower values which were still significantly above baseline (Fig. 13). Similar reticulocyte responses were seen for the other 2 groups of patients, and in the CAPD patient group there was a significant difference between the results for the "responders" and those for the "poor responders" (Fig. 14).

(i v) MCV

In all 3 groups there was no significant change in the MCV during the first 4 months of EPO therapy (Group 1: 91.3±2.4 fl before treatment; 90.8±6.0 fl at 4 weeks; 92.3±6.4 fl at 8 weeks; 94.8±5.3 fl at 12 weeks; 93.5±3.3 fl at 16 weeks), (Group 2: 89.5±5.7 fl before EPO; 89.3±5.4 at 4 weeks; 89.6±5.5 at 8 weeks; 90.0±6.7 fl at 12 weeks; 90.3±7.6 at 16 weeks), (Group 3: 91.7±8.7 before EPO; 93.5±8.9 at 4 weeks; 94.6±8.5 fl at 8 weeks; 92.1±7.3 fl at 12 weeks; 93.1±9.9 fl at 16 weeks). This was despite the significant reticulocytosis described previously.

(v) MCHC

In group 1 patients, no change in MCHC was seen over the first 4 months of EPO treatment (32.6±1.9 g/dl before EPO; 33.4±1.0 g/dl at 4 weeks; 34.2±2.0 g/dl at 8 weeks; 33.2±1.1 g/dl at 12 weeks; 33.4±1.0 g/dl at 16 weeks). In contrast, however, significant falls in this parameter were observed in the other two groups of patients (Fig. 15). The reason for this apparent discrepancy is not clear. It is likely, however, that the fall in MCHC seen in Groups 2 and 3 is caused by the development of mild functional iron deficiency.

(vi) White cell and platelet counts

No significant changes were observed in either the white cell count or the platelet count during EPO therapy in any of the patient groups, values being consistently within the normal ranges (data not shown).

(vii) Serum ferritin

Ferritin levels fell dramatically in all groups of patients during the first few weeks of EPO (Fig. 16). The median pre-treatment value for Group 1 patients was 126 µg/l (range 33-1573 µg/l; normal 15-300 µg/l); this decreased to 85 µg/l (range 12-1126 µg/l) after 4 weeks, 54 µg/l (range 7-1089 µg/l) after 8 weeks, 56 µg/l (range 23-1023 µg/l) after 16 weeks, and 53 µg/l (range 15-733 µg/l) after 1 year of treatment. Five patients, however, required intravenous iron supplementation (Imferon; Fisons Pharmaceuticals, Loughborough, UK) at a dose of 2 ml (100 mg elemental iron) weekly for 4 weeks at around 6-8 weeks of EPO to maintain an adequate iron supply. Similar results were obtained for Group 2 patients [median 130 µg/l (range 28-715 µg/l) before EPO; 65 µg/l (range 22-337 µg/l) at 16 weeks], and Group 3 patients [median 150 µg/l (range 45-462 µg/l) before EPO; 33 µg/l (range 10-152 µg/l) at 16 weeks]. No significant changes in serum B_{12} or folate were seen in any of the groups during EPO therapy.

(viii) Serum iron/TIBC/transferrin saturation

Excluding the 2 patients who were grossly iron-overloaded before starting EPO (serum ferritin >1500 µg/l) and who had pre-treatment transferrin saturations of 75.3% and 79.6% respectively, the mean transferrin saturation for Group 1 patients was 21.6±6.5% before EPO, falling to 14.9±6.5% at 6 weeks ($p<0.01$). Following IV iron supplementation in five patients the transferrin saturation improved to 26.8±22.9% at 8 weeks and 34.0±26.4% at 16 weeks (Fig. 17). Similar results were obtained from the other two patient groups.

(i x) Intravenous iron requirements

Despite all patients being prescribed oral iron supplementation, a large number required intravenous iron to maintain a transferrin saturation above 20%. This was most evident in those who had a starting serum ferritin level below 100 µg/l (Table 5), but many patients who had adequate iron stores (ferritin 100-300 µg/l), and even 3 patients who had increased iron stores (ferritin 300-1000 µg/l) before EPO, needed IV iron. Neither of the 2 patients who were grossly iron-overloaded prior to EPO required intravenous iron supplementation (Table 5).

(x) Biochemical parameters

There were no significant changes in plasma sodium, potassium, bicarbonate, urea, creatinine, or serum calcium, phosphate, total protein, albumin, bilirubin, alkaline phosphatase, gamma glutamyl transferase, aspartate transaminase, or uric acid levels in any of the patient groups throughout EPO treatment. Three of the 38 treated patients (2 haemodialysis, 1 CAPD) had transient individual increases in plasma potassium during the acute correction of their anaemia; these settled rapidly with further dietary advice.

(x i) Ferrokinetic studies

In the eight CAPD patients in whom ferrokinetic measurements were performed before EPO and after achieving their target haemoglobin concentration, there was an increase in the mean (±SD) marrow iron turnover from 78.6±25.1 to 140.1±44.5 µmol/l blood/day; p=0.013 (Fig. 18). Similar rises were observed in the red cell iron turnover (61.8±22.7 to 117.9±43.6 µmol/l blood/day; p=0.012) and the non-erythroid iron turnover (25.5±12.6 to 45.5±15.7 µmol/l blood/day; p=0.026). The degree of ineffective erythropoiesis decreased from 23.1±8.2 to 16.0±6.8% (p=0.05), but there was no change in the mean red cell life-span (56.0±22.5 to 47.3±16.7 days; p=0.299) (Fig. 18).

(xii) Blood volume studies

The red cell volume in the 8 CAPD patients rose from 594.6±115.1 ml before EPO to 1049.0±297.3 ml (p=0.003) after reaching the target haemoglobin concentration (Fig. 19). This was associated with a compensatory fall in the mean plasma volume from 2991.7±588.5 ml to 2521.3±600.9 ml (p=0.005) such that the circulating whole blood volume remained unchanged (3586.3±660.1 ml before EPO to 3570.3±831.2 ml at target Hb; p=0.91) (Fig. 19). There was a reasonable correlation between measurement of haemoglobin concentration and red cell volume both before and after EPO treatment (r=0.801) (Fig. 20).

DISCUSSION

The results presented in this chapter confirm the efficacy of recombinant human erythropoietin in the treatment of renal anaemia. Thirty of the 38 patients reported here achieved the desirable target haemoglobin concentration, and an additional 4 patients may have reached this had treatment not been discontinued prematurely due to renal transplantation in 3 and death in 1. Thus, only 4 patients definitely failed to show a complete response to EPO. In two of these, chronic recurrent respiratory infection was evident, and in both cases EPO was discontinued after several months. In a further patient, moderately severe marrow fibrosis was present on trephine biopsy and the highest haemoglobin concentration he achieved during a 2-year treatment period was 9.0 g/dl (5.5 g/dl pre-EPO). The remaining patient turned out to have metastatic carcinoma of the breast which was not known at the time of commencing EPO. She therefore had the anaemia of malignancy in addition to renal anaemia, and this did not respond to the doses of EPO given.

The two major factors identified in this study which impaired the haematological response to EPO were functional iron deficiency and acute or chronic infection. The former is defined as a state in which the iron supply to the erythron is inadequate to support the needs for erythropoiesis [123,124,125]. This may occur in the presence of either deficient or replete body iron stores. The condition is characterised by a low

transferrin saturation (less than 20%), and on the experience gained in this study and elsewhere, is corrected most effectively by administration of intravenous iron [123,124,125]. Chronic infection has been reported to suppress the response to EPO in other studies. Winearls et al. [90] described two cases in which this was evident, and Adamson & Eschbach [126] reported a case of tuberculous osteomyelitis of the hip which responded to EPO only after treatment of the infection. Other factors which may influence responsiveness to EPO are discussed in Chapter 7 of this thesis.

There was considerable variation among different patients in their initial response to the same starting dose of EPO. This was particularly evident in the Group 1 patients in whom the haemoglobin concentration varied from 6.8 to 13.4 g/dl after 8 weeks of EPO 240 U/kg/wk (pre-treatment Hb ranging from 5.6 to 7.5 g/dl). Consistent with this variability in the sensitivity of response to EPO was the fact that the maintenance dosages after 12 months of treatment showed a nine-fold variation from 85 to 720 U/kg/wk. The reasons for this are almost certainly multifactorial and are discussed in greater detail in Chapter 7.

It was of interest to find that the haemoglobin response in the two groups of patients treated subcutaneously was very similar to that in the group of patients receiving intravenous therapy. This was despite the IV-treated group receiving twice the starting dose of EPO. These results suggest that the pharmacodynamics of subcutaneous EPO are better than for IV EPO, almost certainly due to the fact that serum levels are maintained for a longer period after subcutaneous administration. It also suggests that the high pharmacological peak serum levels obtained following IV EPO (some 100 times greater than endogenous levels) are not required for its therapeutic efficacy. Although, as with IV EPO, the maintenance doses of SC EPO at 1 year show some variability, the dosages are generally lower for SC than for IV administration. This has obvious cost-saving implications when one is dealing with a drug as expensive as EPO.

The efficacy of subcutaneously-administered EPO has been confirmed in other published studies [114,115,116,117,118,119,127,128]. Bommer et al. [114] showed that a 50% reduction in dose could be achieved at optimal haemoglobin

concentration by switching from intravenous to subcutaneous administration. Stevens et al. [117] treated 12 CAPD patients with subcutaneous EPO and obtained a brisk response to a starting dose of 100 or 150 U/kg thrice weekly. The dose was then reduced to 12.5-50 U/kg (median 25 U/kg) thrice weekly to maintain a haemoglobin concentration between 11.0 and 11.5 g/dl. A similarly rapid response was obtained in another study of five transfusion-dependent children on CCPD in whom the haematocrit increased from 22% to 33% after only 3 weeks' treatment with EPO given SC at an initial dose of 150 U/kg thrice weekly [119]. A much lower SC dose of 50 U/kg twice weekly was used by Steinhauer et al. [118] to treat eight adult CAPD patients. In pre-dialysis patients, similar responses were achieved with 150 U/kg thrice weekly intravenously and 100 U/kg thrice weekly subcutaneously [116]. The lowest weekly dose of EPO reported to be effective was also given via the subcutaneous route, in a study by Granolleras et al. [115]. They found that an adequate haemopoietic response was obtained in haemodialysis patients with SC administration of only 14 U/kg daily. Thus, the subcutaneous route appears to be gaining popularity not only in CAPD patients [117,118,127,128] but also in haemodialysis subjects [114,115,129], and the evidence to date suggests that lower doses of EPO may be used when given by this route.

The ferrokinetic studies confirm the expected increase in marrow erythropoietic activity after EPO, showing two-fold increases in the turnover of both marrow and red cell iron. Thus, the rise in haemoglobin concentration and red cell volume was due to increased marrow red cell production since the mean red cell life-span did not change. These findings are in contrast to those described in a separate erythrokinetic study by Hughes et al. [128] who showed a slight improvement in red cell survival after EPO. The reason for this discrepancy is not clear at the present time, although in the latter study the haemoglobin concentration at the point when the improved red cell survival was documented was higher than in the present study. The marginal decrease in ineffective erythropoiesis found in the present study is an encouraging finding which may also contribute to the rise in red cell mass.

The increase in red cell volume with a concomitant decrease in plasma volume such that the circulating whole blood volume remains the same has also been reported in haemodialysis patients by Cotes et al. [130]. In the present study, no specific attempt was made to remove extra fluid and thus contribute to the change in plasma volume.

In conclusion, the results of the present study confirm the efficacy of EPO in treating the anaemia of end-stage renal disease. Many factors can influence the response to therapy which can vary considerably between different patients. On the basis of the preliminary data reported here, it would appear that the subcutaneous route may be preferable for treating both haemodialysis and CAPD patients, allowing lower maintenance dosages and considerable cost savings in the long term.

Table 2 Demographic details of Group 1 patients: 10 haemodialysis patients treated with an initial dose of 240 U/kg/wk IV

Initials	Age (yrs)	Sex	Weight (kg)	Diagnosis	Duration of HD (yrs)	Previous transplants	Transfusions since starting HD (units)	Starting Hb (g/dl)
SW	34	F	39	PN/reflux nephropathy	5.4	1	19	6.1
HL	26	M	73	PN/reflux nephropathy (single kidney)	3.5	0	17	6.3
BB	38	F	64	Chronic GN	0.6	0	4	6.4
RP	48	M	55	Hypertensive nephropathy	1.1	0	8	6.1
EE	51	F	57	Chronic GN	14.7	2	21	7.5
SR	22	M	61	PN/interstitial nephritis	8.2	3	63	6.2
AF	53	M	85	Congenital renal hypoplasia	2.4	0	2	7.0
CL	52	M	74	GN (membranous)	1.6	0	6	5.7
AG	75	M	72	GN (? membranous)	1.4	0	45	5.6
YJ	33	F	47	PN/interstitial nephritis	4.8	1	35	6.1
Mean: (±SD):	43.2 (±15.7)	6M 4F	62.7 (±13.8)		4.4 (±4.3)		22.0 (±20.0)	6.3 (±0.57)

PN = pyelonephritis

GN = glomerulonephritis

Table 3 Demographic details of Group 2 patients: 15 CAPD patients treated with an initial dose of 120 U/kg/wk SC

Initials	Age (yrs)	Sex	Weight (kg)	Diagnosis	Duration of CAPD (yrs)	Previous transplants	Transfusions since starting CAPD (units)	Starting Hb (g/dl)
LR	31	F	55	Henoch Schonlein purpura	4.5	3	15	5.1
WH	64	M	70	Obstructive uropathy	2.2	0	5	5.8
DP	23	M	49	PN/interstitial nephritis	8.2	2	33	5.5
DH	66	F	70	CRF ? cause	5.8	0	0	5.6
DD	69	F	96	Hypertensive/RVD	3.8	0	17	6.1
MJ	63	F	45	CRF ? cause	3.3	0	0	6.6
MD	50	F	44	PN/interstitial nephritis	5.2	0	4	7.7
FA	28	F	55	Reflux nephropathy	4.0	1	3	5.8
NB	74	F	52	CRF ? cause	1.7	0	0	7.2
JJ	66	M	77	Chronic GN	4.4	0	2	6.8
MF	67	F	59	CRF ? cause	1.9	0	0	5.7
LE	34	F	50	Post-partum	1.9	1	10	4.8
MM	38	F	53	Post-partum	2.4	0	0	6.9
MN	55	F	61	Hypertensive/RVD	3.8	0	6	6.9
AR	43	F	54	Chronic GN	4.2	0	3	7.0
Mean: (±SD):	51.4 (±17.2)	3M 12F	59.3 (±13.8)		3.8 (±1.7)		6.5 (±9.1)	6.2 (±0.84)

PN = pyelonephritis CRF = chronic renal failure

GN = glomerulonephritis RVD = renovascular disease

Table 4 Demographic details of Group 3 patients: 13 haemodialysis patients treated with an initial dose of 120 U/kg/wk SC

Initials	Age (yrs)	Sex	Weight (kg)	Diagnosis	Duration of HD (yrs)	Previous transplants	Transfusions since starting HD (units)	Starting Hb (g/dl)
HE	19	F	46	CRF ? cause	1.1	0	10	6.7
PF	24	M	78	PN/reflux nephropathy (single kidney)	1.1	0	4	7.5
MH	30	F	44	PN/reflux nephropathy	1.1	0	2	7.1
MJ	16	F	46	PN/interstitial nephritis	6.3	1	8	6.4
ChL	28	F	47	Reflux nephropathy	3.6	0	22	6.9
BS	52	M	67	CRF ? cause	0.8	0	2	7.8
HW	64	M	74	CRF ? cause	6.6	0	17	6.7
EdW	59	M	81	Polycystic kidney disease	5.4	0	12	6.0
EiW	32	F	68	PN/reflux nephropathy	0.5	0	12	6.5
NC	29	M	63	Systemic lupus erythematosus	2.2	0	14	7.5
KH	65	M	64	Obstructive uropathy	0.6	0	4	7.4
YP	31	F	48	IgA nephropathy	0.8	0	8	7.5
BH	35	M	36	Congenital obstructive uropathy	0.5	0	4	5.3
Mean: (±SD):	37.2 (±16.9)	7M 6F	58.6 (±14.8)		2.4 (±2.3)		9.2 (±6.1)	6.9 (±0.71)

PN = pyelonephritis

CRF = chronic renal failure

Table 5 Requirements for intravenous iron supplementation during EPO therapy according to pre-treatment serum ferritin levels.

Initial ferritin (µg/l)	No. of patients	No. given IV Fe
<100	17	14
100-300	11	9
300-1000	8	3
>1000	2	0

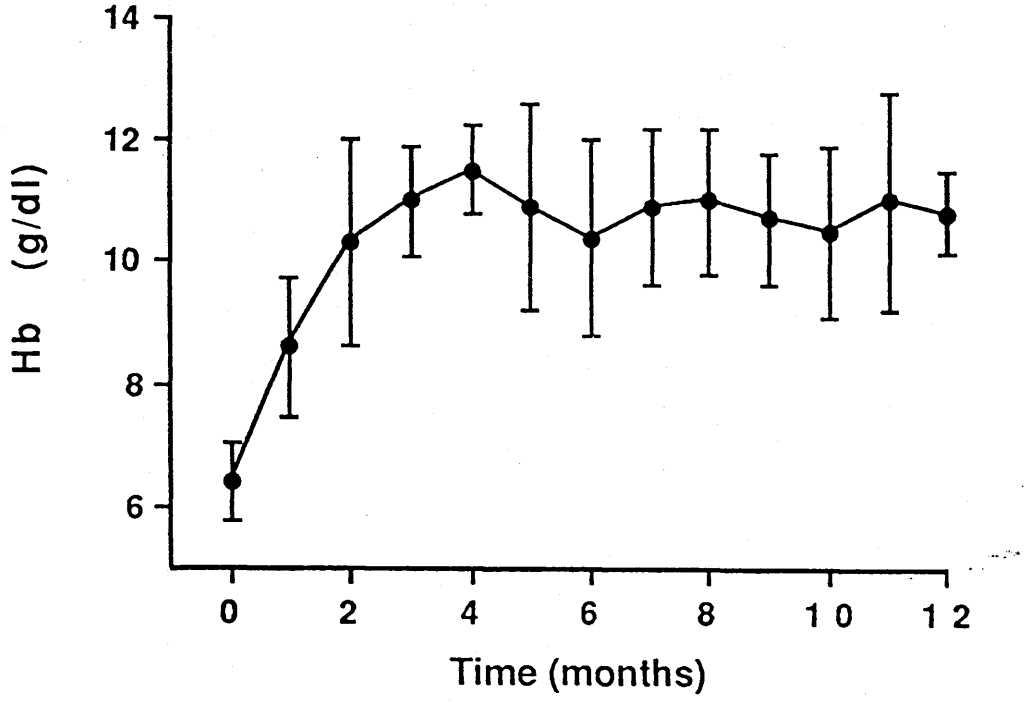


Fig. 5 Haemoglobin response to EPO in 9 haemodialysis patients treated with an initial dose of 120 U/kg IV twice weekly.

Results expressed as means ± SD.

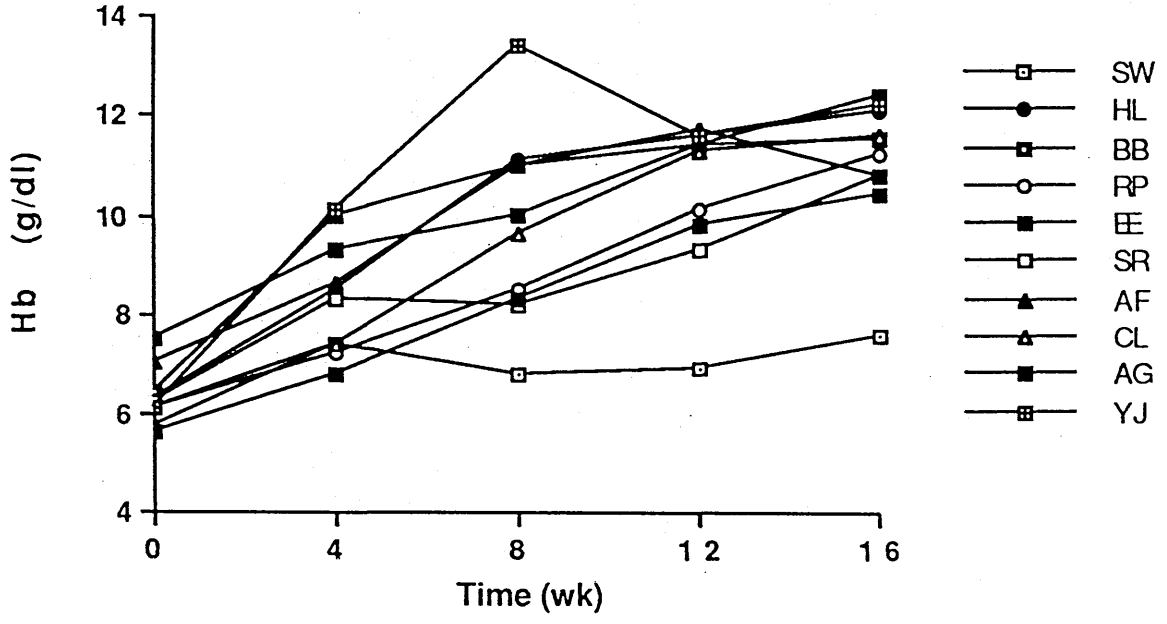


Fig. 6 Variability of haemoglobin response to EPO in 10 haemodialysis patients treated with an initial dose of 120 U/kg IV twice weekly.

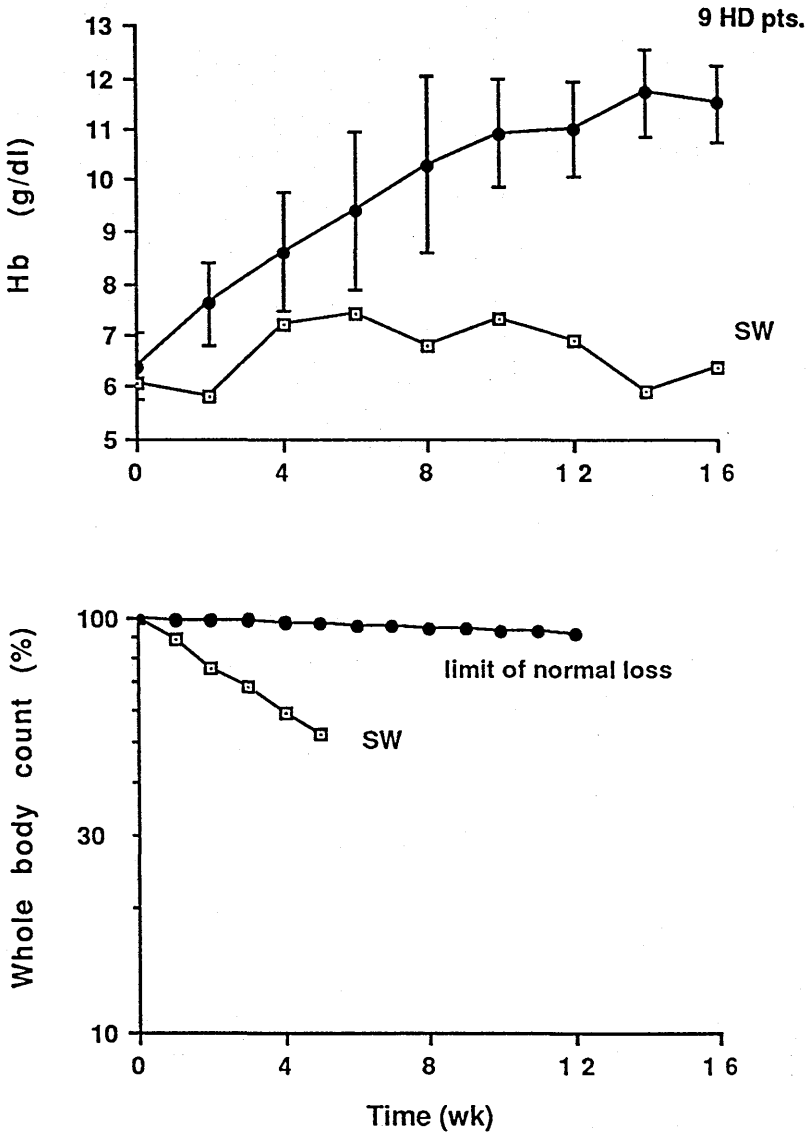


Fig. 7 Impaired haemoglobin response to EPO in a 34-year-old female haemodialysis patient (SW) who was found to have gastrointestinal bleeding. Lower panel shows quantitative ^{59}Fe blood loss studies which were strikingly positive. Upper gastrointestinal endoscopy revealed several gastric erosions as the presumed source of bleeding.

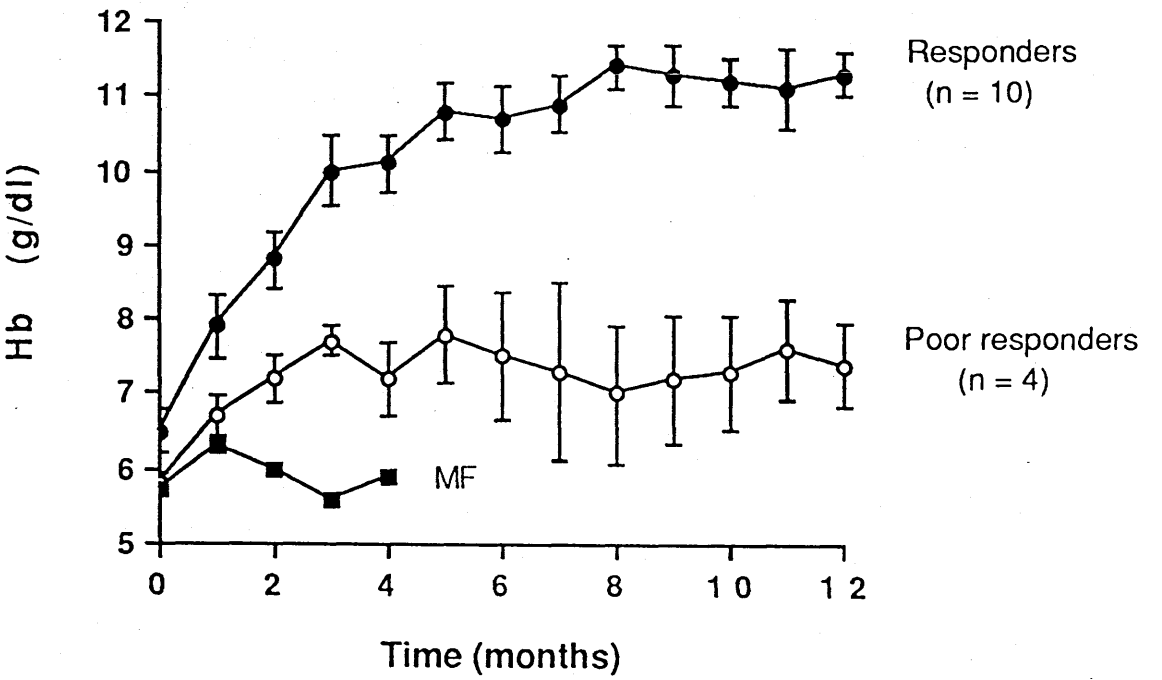


Fig. 8 Haemoglobin response to EPO in 15 CAPD patients treated with an initial dose of 60 U/kg subcutaneously twice weekly.

Results for the 10 responders and 4 poor responders expressed as means ± SD. Also shown is the lack of haemoglobin response in a 67-year-old female (MF) who was subsequently found to have occult carcinomatosis of the breast (see text for details).

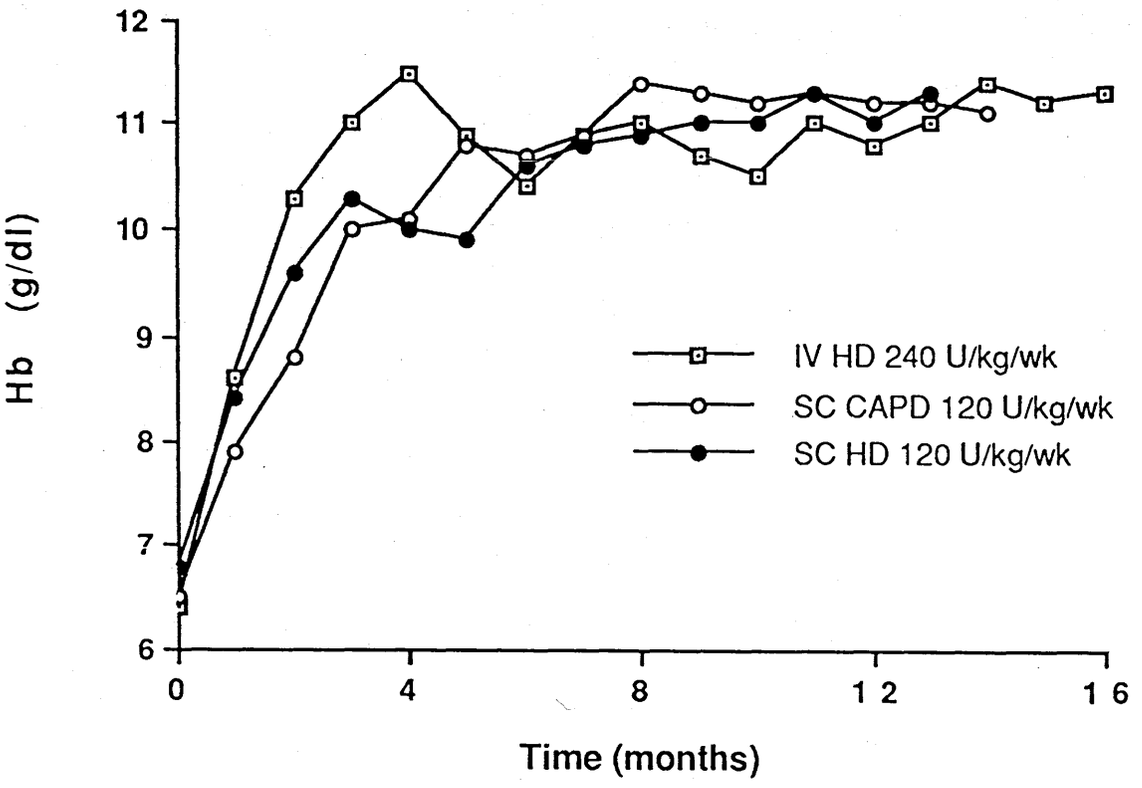


Fig. 9 Mean haemoglobin response to EPO in 3 groups of dialysis patients:- (i) 9 haemodialysis (HD) patients treated intravenously (IV) with 240 U/kg/wk of EPO; (ii) 10 CAPD patients treated with 120 U/kg/wk of EPO given subcutaneously (SC); and (iii) 10 haemodialysis patients treated with 120 U/kg/wk of EPO given subcutaneously.

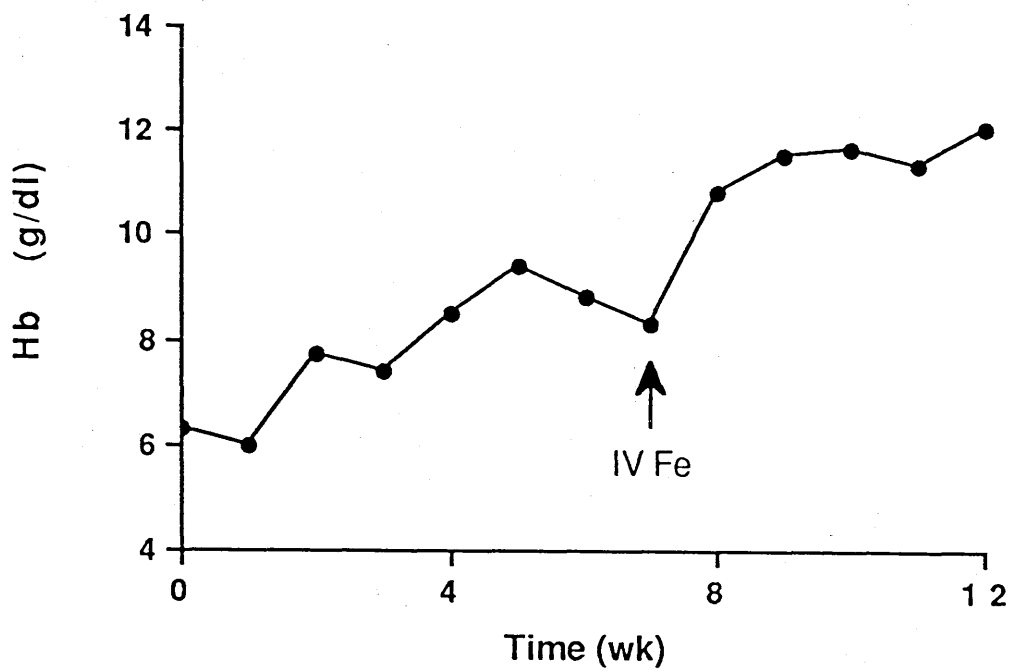


Fig. 10 Impaired haemoglobin response to EPO in a 26-year-old male haemodialysis patient which was restored following intravenous administration of 2 ml iron dextran (Imferon; 50 mg elemental iron/ml) during week 7.

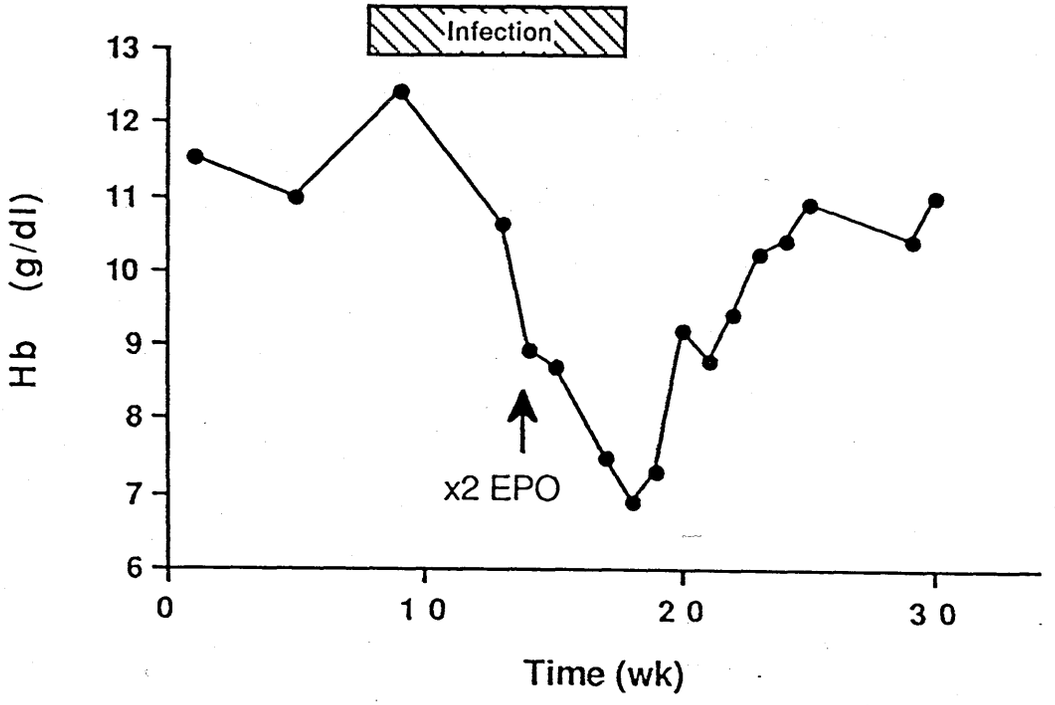


Fig. 11 Loss of the haemoglobin response to EPO in a 52-year-old male haemodialysis patient who developed a severe chest infection after 13 months of treatment. Doubling the EPO dose had no effect, and it was only after the infection had resolved that the haemoglobin response started to recover.

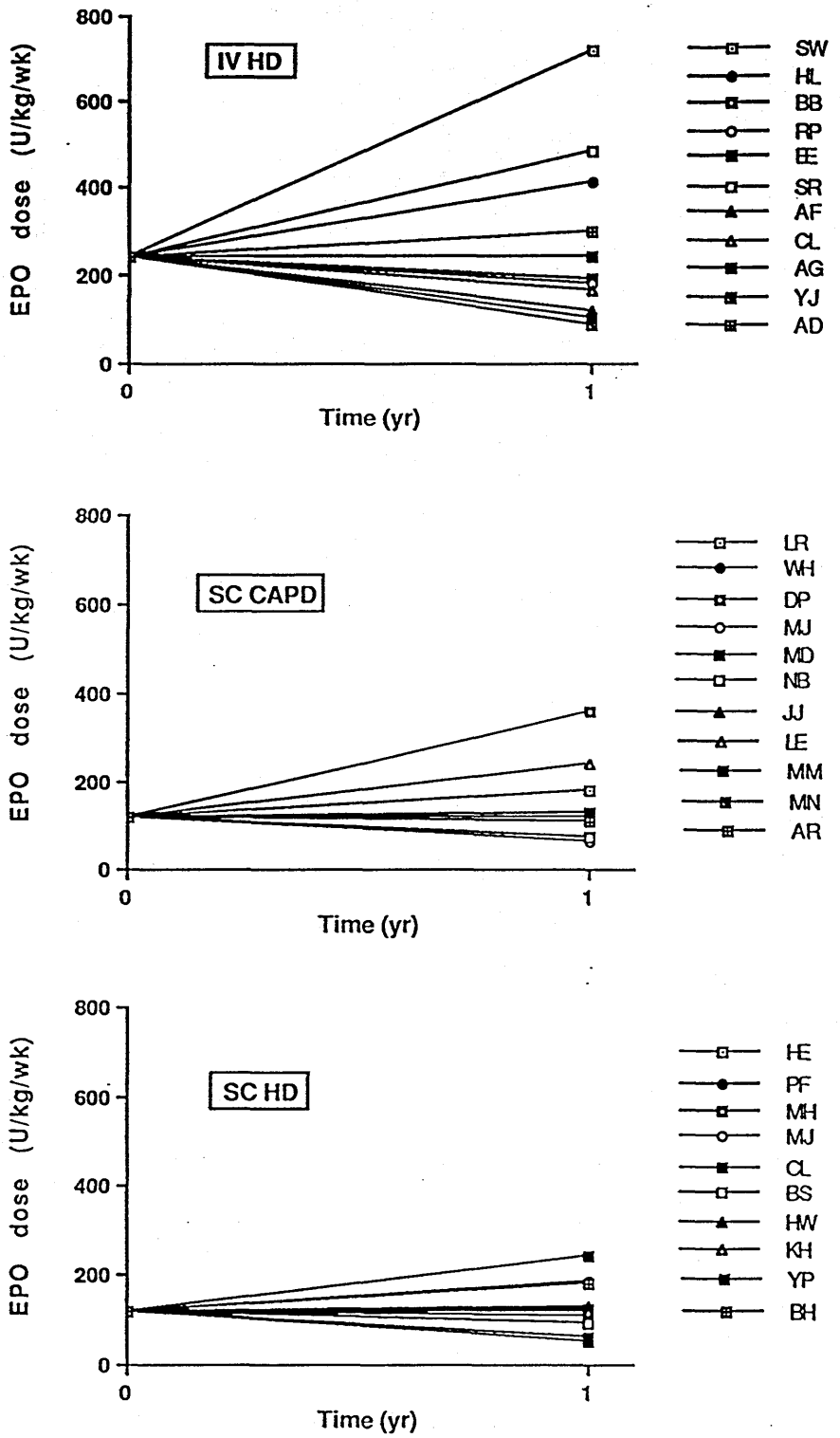


Fig. 12 Maintenance doses of EPO in 3 groups of dialysis patients after 1 year of therapy compared to their starting doses.

Upper panel: Group 1. Haemodialysis patients treated with IV EPO at an initial dose of 240 U/kg/wk.

Middle panel: Group 2. CAPD patients treated with SC EPO at an initial dose of 120 U/kg/wk.

Lower panel: Group 3. Haemodialysis patients treated with SC EPO at an initial dose of 120 U/kg/wk.

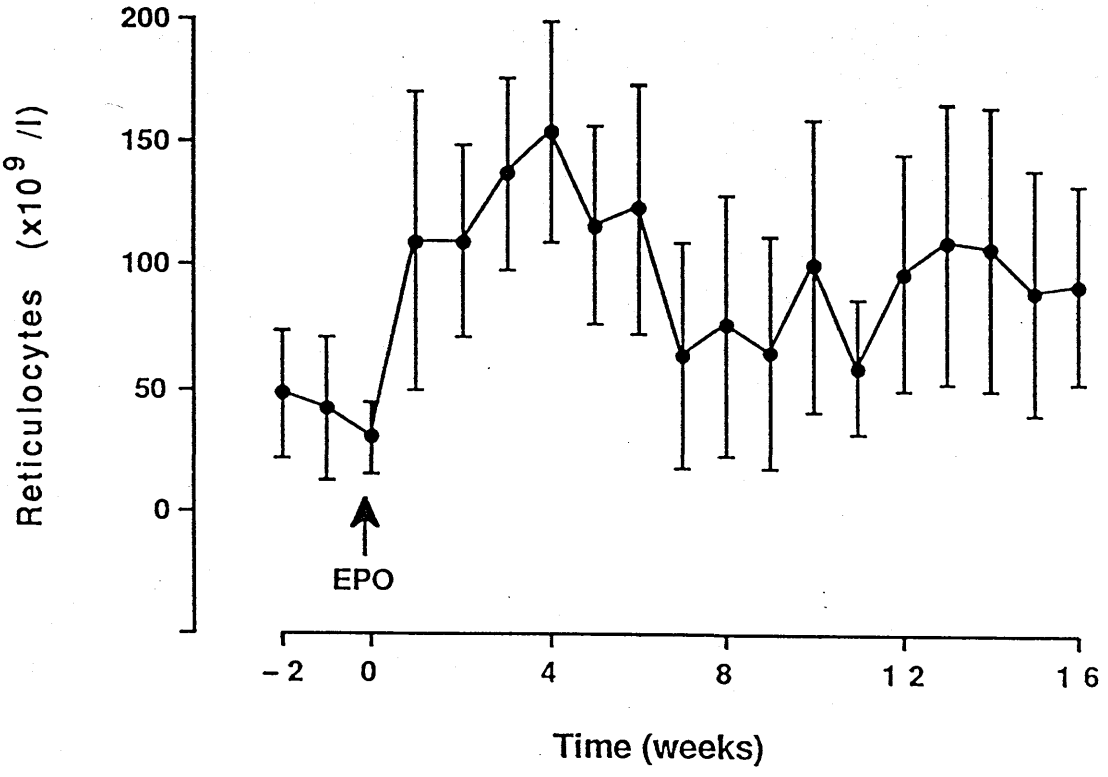


Fig. 13 Reticulocyte response to EPO in 10 haemodialysis patients treated with an initial dose of 120 U/kg IV twice weekly.

Results expressed as means ± SD.

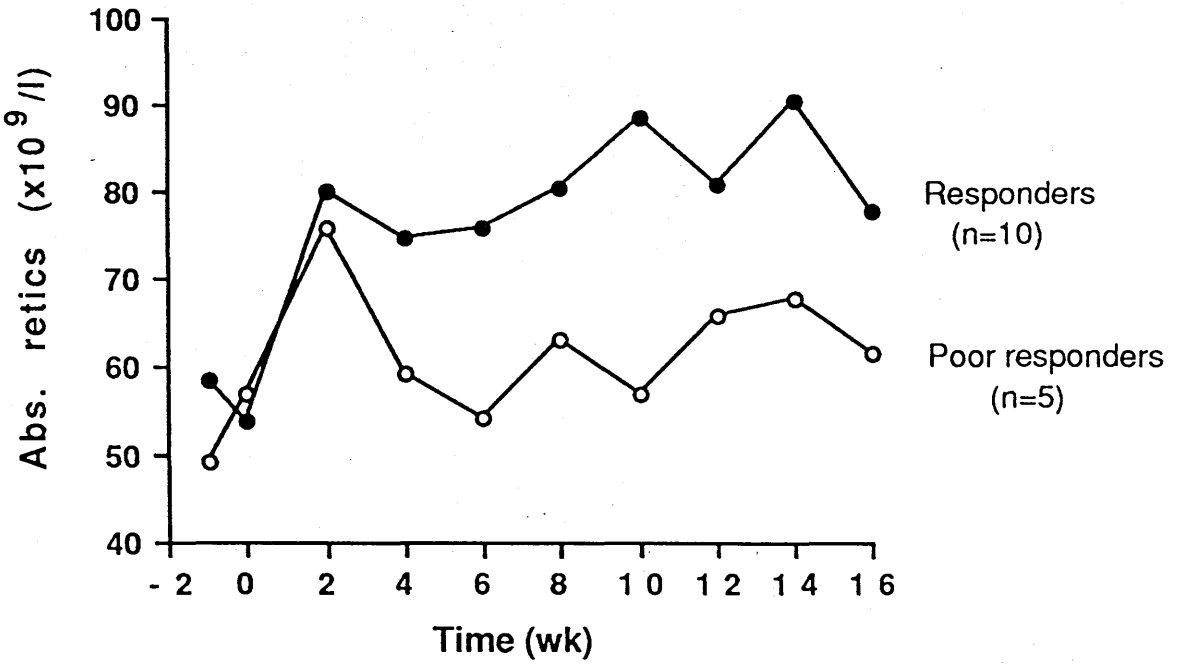


Fig. 14 Mean reticulocyte response to EPO in 15 CAPD patients treated with an initial dose of 60 U/kg subcutaneously twice weekly. Results are shown for the 10 good responders versus the 5 poor or non-responders (see text for details).

Abs. retics. = absolute reticulocyte count.

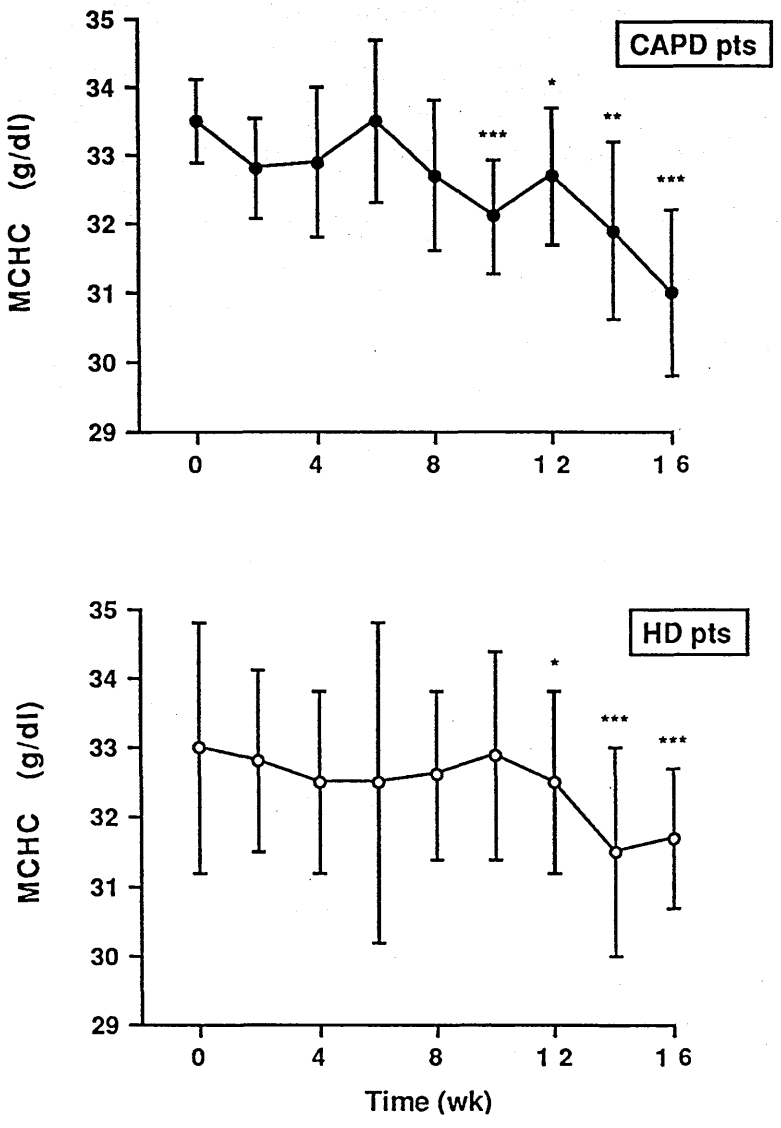


Fig. 15 Effect of EPO on the mean cell haemoglobin concentration (MCHC) in 2 groups of dialysis patients treated with an initial dose of 60 U/kg subcutaneously twice weekly.

Upper panel = CAPD patients.

Lower panel = Haemodialysis patients.

Results expressed as means ± SD.

*p<0.05, **p<0.01, ***p<0.005 compared with baseline.

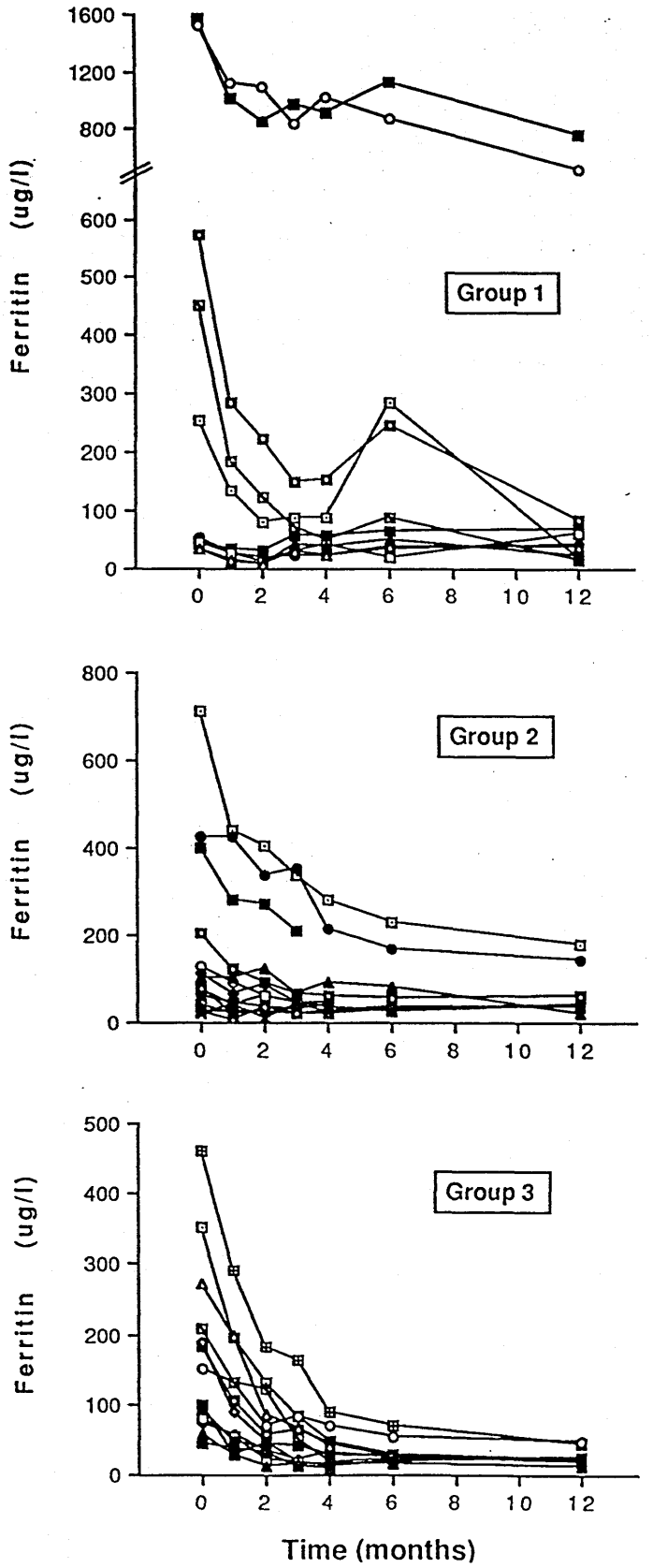


Fig. 16 Effect of EPO on the serum ferritin level in 3 groups of dialysis patients.

Upper panel: Group 1. 10 haemodialysis patients treated with an initial dose of 240 U/kg/wk IV.

Middle panel: Group 2. 15 CAPD patients treated with an initial dose of 120 U/kg/wk SC.

Lower panel: Group 3. 13 haemodialysis patients treated with an initial dose of 120 U/kg/wk SC.

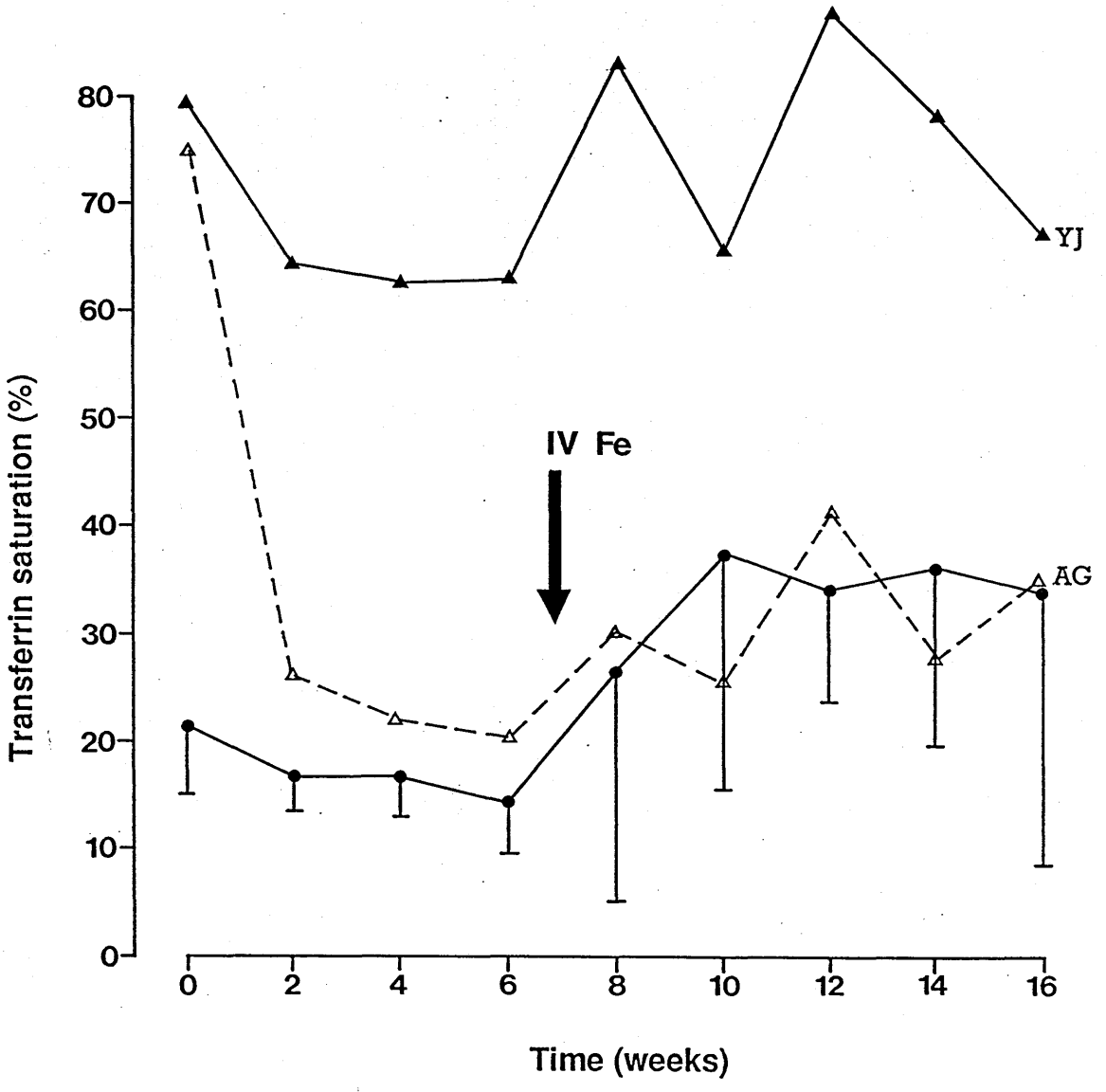


Fig. 17 Effect of EPO on the transferrin saturation in 10 haemodialysis patients treated with an initial dose of 120 U/kg IV twice weekly.

●——● mean ± SD results for 8 of the patients who had no evidence of excessive iron overload at the start of treatment. Five of the patients were given intravenous iron supplementation in the form of iron dextran (Imferon) during week 7.

AG, YJ: two patients who had evidence of excessive iron stores at the start of EPO therapy (initial serum ferritin >1500 µg/l).

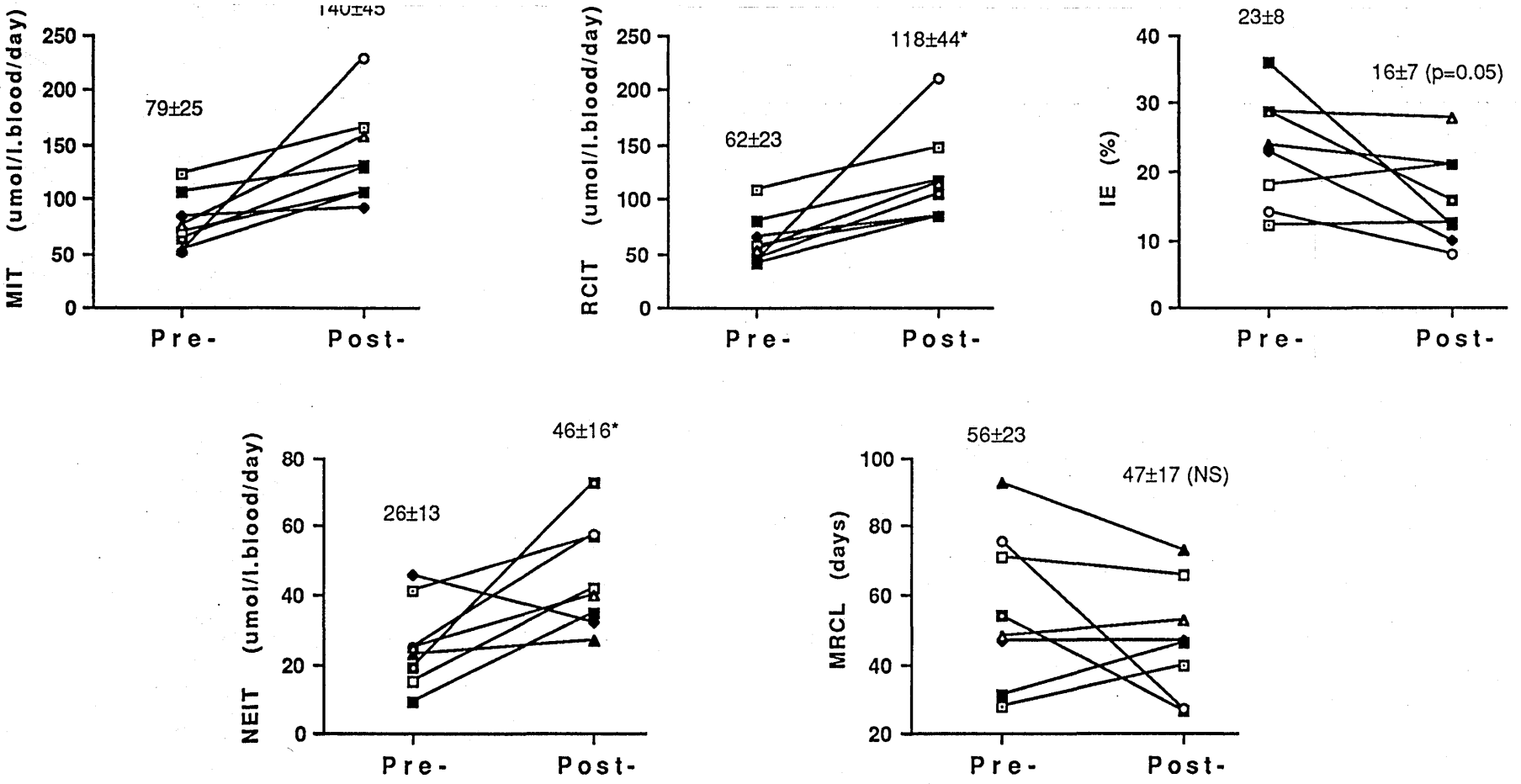


Fig. 18 Ferrokinetic measurements in 8 CAPD patients before and after treatment with EPO 120 U/kg/wk SC. The first assessment was carried out immediately prior to the onset of therapy; the second study was conducted after a haemoglobin concentration of >9 g/dl had been attained. Figures shown represent the means ± SD for each parameter (*$p<0.05$, NS = not significant compared to baseline).

MIT = marrow iron turnover; RCIT = red cell iron turnover; IE = ineffective erythropoiesis; NEIT = non-erythroid iron turnover; MRCL = mean red cell life-span.

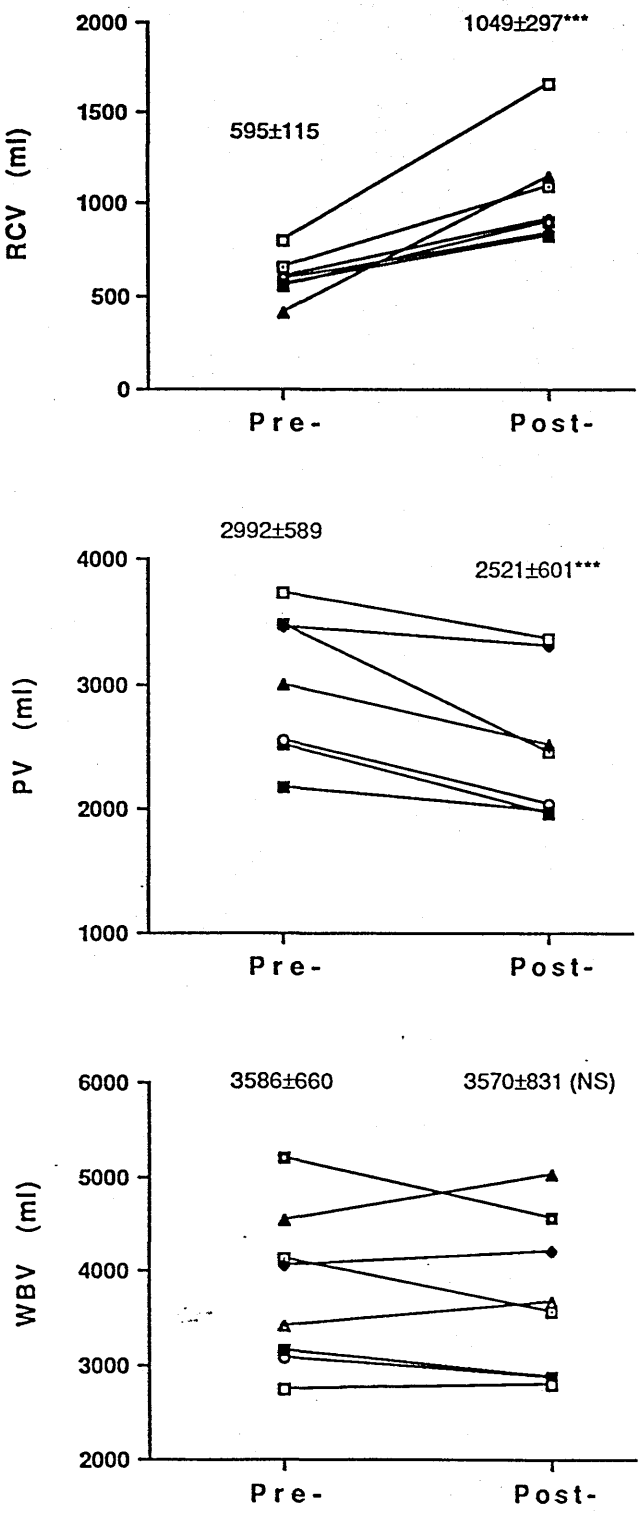


Fig. 19 Blood volume studies in 8 CAPD patients before and after treatment with EPO 120 U/kg/wk SC. The first assessment was carried out immediately prior to the onset of therapy; the second study was conducted after a haemoglobin concentration of >9 g/dl had been attained. Figures shown represent the means ± SD for each parameter (***$p<0.005$, NS = not significant compared to baseline).

RCV = red cell volume; PV = plasma volume; WBV = whole blood volume.

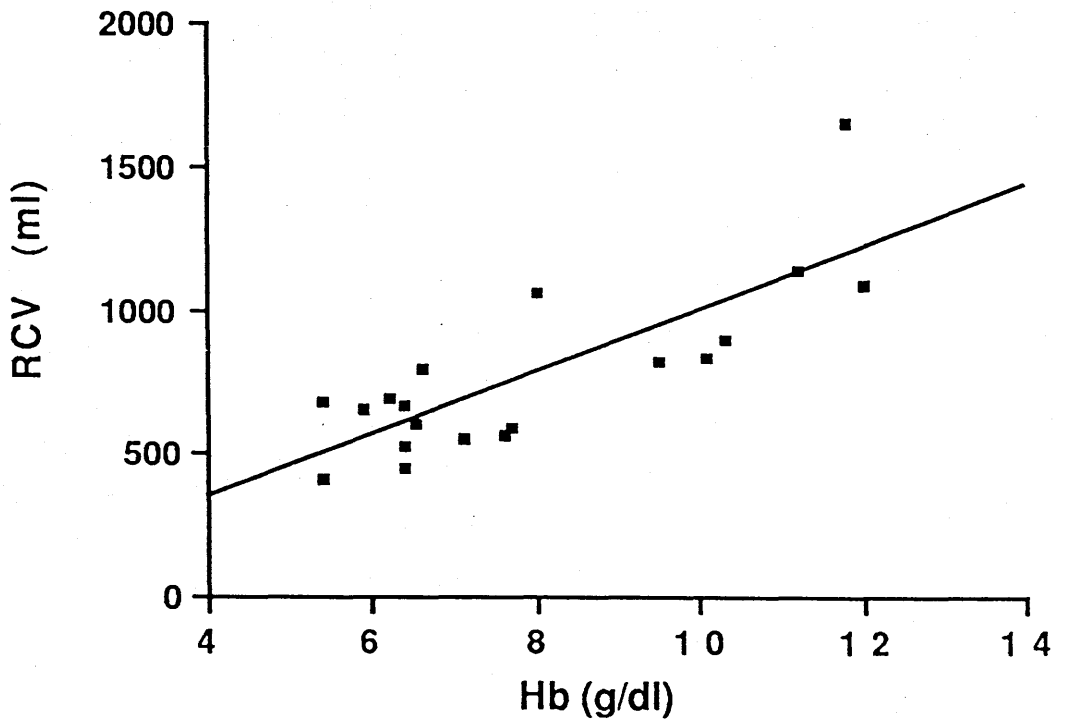


Fig. 20 Correlation between haemoglobin concentration (Hb) and red cell volume (RCV) in 12 CAPD patients before treatment, and 8 CAPD patients after treatment with EPO 120 U/kg/wk SC (r = 0.801).

CHAPTER 3

CARDIORESPIRATORY BENEFITS FOLLOWING CORRECTION OF RENAL ANAEMIA BY ERYTHROPOIETIN

INTRODUCTION

Patients maintained on chronic haemodialysis have a markedly reduced exercise capacity [131,132,133,134] and impaired cardiorespiratory function [135] when compared with age- and sex-matched individuals who are non-uraemic. Various factors may be contributory including hypertension, hypervolaemia, electrolyte imbalance, arteriovenous fistula, calcification, and "uraemic" toxins, but the major determinant appears to be chronic anaemia [134].

Longstanding severe anaemia has profound effects on the cardiovascular system, resulting in an increase in cardiac output, decrease in peripheral resistance due to compensatory vasodilation secondary to tissue hypoxia, and reduced blood viscosity [136,137,138,139,140]. In addition, the consequent reduction in oxygen-carrying capacity of the blood results in impaired oxygen delivery to the myocardium which, in turn, exacerbates myocardial ischaemia in individuals who are already predisposed to coronary artery disease as a result of abnormal lipoprotein profiles and other factors [141,142,143,144,145]. Furthermore, the anaemia of end-stage renal failure is believed to play a major role in the development of left ventricular hypertrophy in patients receiving long-term dialysis [146,147]. This, in turn, appears to be an important independent determinant of survival in such patients [148].

Previous studies have shown that acute correction of uraemic anaemia by red cell transfusion was followed by a normalisation of the changes in cardiac output and total peripheral resistance [149]. It was likely, therefore, that long-term correction of renal anaemia by recombinant human erythropoietin would have major effects on cardiorespiratory function. Furthermore, since cardiovascular complications account for more than half of all mortality in patients with chronic renal failure [150], it is clearly essential that EPO therapy should not exacerbate these problems. While patients receiving EPO have almost universally reported a subjective improvement in well-being and exercise tolerance [151,152,153], there is limited information on the secondary effects of a rise in haematocrit in such individuals, although a recent

investigation showed a short-term improvement in exercise capacity in haemodialysis patients treated with EPO [154].

The study reported in this chapter sought to investigate the long-term effects of EPO therapy on cardiac and respiratory function by means of maximal exercise testing with concomitant respiratory gas analysis and electrocardiographic monitoring, lung function tests, echocardiography, chest radiography, and rheological assessment over a 12-month period.

PATIENTS AND METHODS

Patients

Ten patients on regular haemodialysis were studied. These comprised 6 males, 4 females; the mean age was 43 years (range 22-75 years), and the mean duration on haemodialysis was 4.4 years (range 0.6-14.7 years). The cause of their renal disease was chronic glomerulonephritis (4 patients), chronic pyelonephritis/interstitial nephritis (2 patients), reflux nephropathy (2 patients), hypertensive nephropathy (1 patient), and congenital renal hypoplasia (1 patient). The mean pre-treatment haemoglobin concentration was 6.4±0.63 (SD) g/dl, and all patients had normal serum ferritin (>30 µg/l), vitamin B_{12}, and folate levels, with no obvious cause for anaemia other than their renal failure. Pre-treatment serum EPO levels were all inappropriately low for the degree of anaemia, ranging from 25 to 34 mU/ml. Two patients had a past history of hypertension although no patients were on anti-hypertensive medication at the commencement of the study. One patient (34 years, female) had suffered 2 previous myocardial infarctions during the preceding 2 years and had subsequently undergone a right coronary angioplasty; another patient (75 years, male) had a past history of recurrent transient ischaemic attacks, and a further 2 patients had sustained previous thrombosis of their arteriovenous fistula. No patient was known to have any respiratory disease, although three were regular cigarette smokers.

All patients were treated with EPO (Boehringer Mannheim GmbH, Germany) 120 U/kg given intravenously twice weekly at the end of dialysis. One patient had to be withdrawn from the study after 4 months on EPO due to a substantial fall in her haemoglobin resulting from persistent gastrointestinal bleeding; results on this patient are therefore available up to this point only.

Methods

The following tests were all performed on the day prior to a dialysis session.

Exercise testing. Symptom-limited maximal exercise testing using the Weber treadmill protocol was conducted before, and after 2, 4, 8, and 12 months of treatment with EPO. Respiratory gas analysis during exercise was performed by means of mass spectrometry (Airspec 2000, Airspec Ltd., Biggin Hill, UK) using the argon dilution technique [155]. The duration of exercise and the maximal oxygen consumption were recorded, and the anaerobic threshold determined by the method of Matsumura et al. [156] using a linear regression technique. Blood pressure was measured every 2 minutes using a mercury sphygmomanometer. The heart rate and electrocardiogram were monitored continuously (Marquette Case System, Marquette Electronics, Milwaukee, USA). A standard 12-lead electrocardiogram was recorded after each minute. Myocardial ischaemia was defined as the presence of $\geq$ 0.1 mV horizontal or downsloping S-T segment depression. All electrocardiograms were assessed blind by an independent observer who was unaware of the clinical details.

Echocardiography. Echocardiograms were performed by the same operator using a Hewlett Packard Duplex system (Hewlett Packard, USA) prior to, and after 4, 8, and 12 months of treatment with EPO in 9 patients. Care was taken to ensure the same transducer positioning for measurement of left ventricular (LV) dimensions from the parasternal long-axis view. Left ventricular mass was calculated using the method of Devereux & Reichek [157].

Lung function tests. Standard lung function tests were carried out before, and after 2, 4, and 12 months of EPO therapy in 9 patients. These included measurement of peak flow rate (PFR), forced expiratory volume in one second ($FEV_{1.0}$), and forced vital capacity (FVC) obtained from the flow volume curve using the Transfer Test USA from PK Morgan Ltd. Functional residual capacity (FRC), residual volume (RV), slow vital capacity (VC), and total lung capacity (TLC) were measured by means of a closed circuit helium dilution technique using the same equipment. The transfer factor (TL) was measured by the single-breath carbon monoxide method. The results were presented in two ways: (i) as observed (TL_{obs}), and (ii) corrected to a standard haemoglobin concentration of 14.6 g/dl (TL_{corr}).

Rheological studies. 20 ml of whole blood was withdrawn into an EDTA-coated bottle before EPO therapy was commenced and every subsequent 2 weeks up to 4 months of treatment, and finally after 12 months of treatment. On each occasion, plasma (PV) and serum (SV) viscosities were measured on a Luckham (Burgess Hill, UK) viscometer, and whole blood viscosity (WBV) was determined on a Haake (Karlsruhe, Germany) CV 100 viscometer at 3 shear rates (3 s^{-1}, 30 s^{-1}, and 300 s^{-1}). Results were expressed throughout as mPa sec.

Chest radiology. A standard chest X-ray was performed immediately before EPO treatment and after 6 and 12 months of therapy in 8 patients. The cardiothoracic ratios were determined on each occasion by the same observer who was unaware of the stage of treatment.

Haemoglobin concentration. This was measured weekly on a Technicon (Basingstoke, UK) H1 automated blood count analyser up to 4 months of treatment, and measured monthly thereafter.

Analysis of results

Analysis of variance and the paired t test (two-tailed) were used throughout to compare results at different time intervals, with the exception of the results of the exercise ECG when a Fisher's exact test was employed. Mean values and standard deviations quoted are for the group as a whole.

RESULTS

Haemoglobin response

The mean haemoglobin concentration had risen substantially by 2 months of EPO therapy (6.4±0.63 g/dl before treatment, to 10.3±1.7 g/dl at 2 months); there were further smaller increases to 4 months and the improvement was then maintained but not augmented to the end of the study (11.5±0.74 g/dl at 4 months, 11.0±1.2 g/dl at 8 months, and 10.8±0.68 g/dl at 12 months). Corresponding values for haematocrit were 0.198±0.02 (0 months), 0.299±0.048 (2 months), 0.343±0.03 (4 months), 0.335±0.042 (8 months), and 0.326±0.013 (12 months).

Blood viscosity measurements

Plasma and serum viscosities did not change throughout the first 4 months of EPO treatment. In contrast, there was a gradual rise in whole blood viscosity measured at all three shear rates, the difference being more marked the lower the shear rate [3.16±0.62 to 8.08±1.31 mPa sec (3 s^{-1} shear rate); 2.12±0.29 to 3.83±0.53 mPa sec (30 s^{-1} shear rate); and 1.88±0.29 to 2.84±0.22 mPa sec (300 s^{-1} shear rate)]. These results indicate a 2.6-, 1.8-, and 1.5-fold rise in WBV over the first 4 months for the 3, 30, and 300 s^{-1} shear rates respectively. There was no further increase in the viscosity measurements after 4 and 12 months of EPO therapy.

Exercise testing

Exercise duration. The duration of exercise achieved rose in all 10 patients after 2 months of treatment (mean 13.2±5.5 to 20.0±6.2 minutes; $p<0.0001$) (Fig. 21). This improvement was maintained but not further increased on repeat testing at 4 months (21.8±6.8 minutes) when the mean haemoglobin concentration was higher, 8 months (21.2±6.7 minutes), and 12 months (20.7±7.5 minutes).

Maximal oxygen consumption. This rose in 7 of the 10 patients after 2 months of EPO treatment with a mean increase from 19.1±7.0 to 25.0±6.7 ml/min/kg ($p<0.01$). Results obtained at 4 months (25.5±5.1 ml/min/kg), 8 months (24.1±7.0 ml/min/kg), and 12 months (22.8±4.8 ml/min/kg) were unchanged from those obtained at 2 months (Fig. 22).

Anaerobic threshold. Using the method of Matsumura et al. [156], the anaerobic threshold could be determined in 9 of the patients before EPO therapy. A significant increase in anaerobic threshold was observed after 2 months' treatment with EPO with a mean increase from 11.7±3.6 to 15.4±4.8 ml/min/kg ($p<0.0005$). This was maintained over the subsequent 10 months, with the mean response at 4 months (14.6±3.3 ml/min/kg), 8 months (14.3±3.1 ml/min/kg), and 12 months (13.9±2.6 ml/min/kg) being similar to that obtained at 2 months (Fig. 23).

Exercise ECG. Eight out of 10 patients had one or more areas of significant S-T segment depression prior to EPO therapy (Table 6); only one of these was known to have coronary artery disease and experienced anginal symptoms. The abnormalities resolved in all but one patient (BB) after 2 months of treatment ($p=0.019$; Fisher's exact test) despite a significant increase in the duration of exercise performed (an example is shown in Fig. 24). After 4 months of treatment this patient (BB) too had complete resolution of her S-T segment depression. Another patient (HL), however, had some recurrence of ischaemia at 4 months, though to a lesser extent than during his pre-

treatment assessment. This S-T segment depression occurred only after 20.7 minutes of exercise which represented considerably more physical effort than was achieved prior to EPO therapy (11.9 minutes). At 8 months, only one of the 9 patients who had an exercise ECG performed showed any evidence of myocardial ischaemia, and at 12 months all 9 patients were free of ECG evidence of ischaemia. No significant cardiac arrhythmias were noted during exercise at any stage of testing, and there were no new ischaemic ECG changes or symptoms in the two patients who were free of these before EPO therapy was commenced.

Echocardiography

There was a significant progressive reduction in LV mass from 354±169 g before EPO to 311±149 g after 4 months' treatment, 291±112 g after 8 months', and 251±95 g after 12 months' therapy ($p<0.01$) (Fig. 25). This was more obvious the greater the degree of left ventricular hypertrophy at baseline (see example in Fig. 26). Left ventricular internal dimensions in systole and diastole were not significantly altered by EPO therapy, though there was a downward trend (3.9±1.0 versus 3.6±1.2 cm, and 5.3±1.1 versus 4.9±1.2 cm at 0 and 12 months respectively) (Fig. 27).

Chest radiology

Four of 8 patients showed a significant (>10%) reduction in cardiothoracic ratio (CTR) after 6 months of EPO (Fig. 28). The mean CTR decreased from 0.51±0.04 to 0.46±0.04 over this period ($p<0.05$), and this improvement was maintained on repeat examination at 12 months (0.46±0.04). No other changes in the chest X-ray were noted.

Lung function tests

There was a significant increase in CO transfer (TL_{obs}) in all patients after 2 months of EPO (mean 15.5±2.9 to 18.6±3.7 ml/min/mmHg; $p<0.005$) (Fig. 29). A further increase to 21.8±3.3 ml/min/mmHg ($p<0.001$) was observed at 4 months,

which was maintained on re-testing at 12 months (21.4±4.5 ml/min/mmHg). However, there was no significant change when values were corrected to a standard haemoglobin concentration (TL_{corr}). There were likewise no significant changes in the other parameters of lung function (PFR, $FEV_{1.0}$, FVC, FRC, RV, VC, or TLC) over the 12 months of treatment.

Blood pressure

All patients were normotensive at the start of EPO therapy (mean MAP = 102±19 mmHg) and none were receiving anti-hypertensive medication. After 4, 8, and 12 months of EPO the mean MAP was unchanged (108±20, 110±25, and 107±17 mmHg, respectively); however 2 patients required hypotensive therapy. Patient RP commenced atenolol at week 7; this was subsequently discontinued at week 12 and he remained normotensive thereafter. Patient EE required triple anti-hypertensive therapy to control her blood pressure and she remained on atenolol and prazosin at 12 months.

DISCUSSION

The data presented here provided, for the first time, information on the long-term effects of EPO on cardiac and respiratory function [158]. In particular, this study has demonstrated beneficial responses to the recombinant hormone in terms of exercise capacity, exercise-induced myocardial ischaemia, left ventricular mass, cardiac size as assessed by chest X-ray, and lung function tests, which were maintained over at least 12 months once a stable haemoglobin concentration was reached.

Numerous clinical trials have demonstrated an almost universal haemopoietic response to EPO therapy in dialysis patients [1,2,3,4,90,91,92,93,159]. Many of these studies document an increase in patient well-being, quality of life, and subjective improvement in physical capacity [151,152,153]. There is, however, a relative paucity of published information on the effect of EPO on cardiac or respiratory function. Although an improved peripheral oxygen delivery resulting from partial correction of

renal anaemia with EPO would be expected to be beneficial, other related changes might be less so. Associated with the rise in haematocrit is an exponential increase in whole blood viscosity (see Chapter 4) which, in turn, results in an increased peripheral resistance [160], and which could adversely affect coronary blood flow. Peripheral resistance is known to increase after EPO therapy [161,162,163,164]. Furthermore, studies in hypertensive patients have suggested that there is a strong relationship between whole blood viscosity and the development of left ventricular hypertrophy which is independent of blood pressure levels [165]. Other implications of an increased blood viscosity have been summarised in a recent review article [160].

This study was therefore aimed at examining the effects of partial correction of renal anaemia on cardiac and respiratory function in haemodialysis patients utilising, in particular, maximal exercise testing with respiratory gas analysis which has been shown to be one of the best methods available for testing cardiorespiratory function [166,167]. Haemodialysis patients are known to have impaired exercise capacity [131,132,133,134], largely due to anaemia [134]. The work reported in this chapter has confirmed Mayer and colleagues' results showing an early improvement in exercise tolerance, maximal oxygen consumption, and anaerobic threshold after EPO treatment [154]. In addition, the results presented here show that these benefits are maintained in the longer term. This is almost certainly due to the better oxygen-carrying capacity of the blood which would also explain the substantial improvements in CO transfer (TL_{obs}) found in the present study. Cotes et al. [168] previously showed that treatment of iron deficiency anaemia resulted in a rise in CO transfer; the present study provides confirmatory evidence that this also occurs with EPO therapy.

Of even greater interest is the reversal of exercise-induced myocardial ischaemia after EPO therapy in all 8 patients who had evidence of S-T depression on exercise ECG prior to treatment. This suggests that the improved oxygen delivery to the myocardium is outweighing the possible deleterious effect of the increased blood viscosity on coronary blood flow. Furthermore, this amelioration of cardiac ischaemia was achieved despite a considerable increase in workload (a doubling of the exercise time

results in a disproportionately greater increase in work output). Two of the 8 patients with cardiac ischaemia had symptomatic angina prior to EPO; both noted a subjective improvement in their symptoms with less frequent utilisation of their sublingual glyceryl trinitrate spray. In the context of current concern about the development of thrombotic complications including myocardial infarction, these results suggest that the benefits outweigh the risks, and that ischaemic heart disease should not be regarded as a contraindication to EPO therapy. Care should still be taken, however, not to raise the haematocrit to too high a level in case the benefit is lost.

Longer term beneficial cardiac effects may also result from EPO therapy. Patients with moderately severe anaemia have a high cardiac output in an attempt to compensate for their reduced oxygen carrying capacity. This results in compensatory hypertrophy of the left ventricle [146,147], a finding which is common in haemodialysis patients and which has recently been shown to be an important independent determinant of survival in such patients [148]. There is good reason to speculate that if the anaemia is corrected with EPO and blood pressure control maintained, progression of left ventricular hypertrophy may be retarded or even partially reversed. A recent study has reported a reduction in LV mass following renal transplantation [169], attributing the effect to an increased haematocrit and closure of arteriovenous fistulae. The decline in estimated LV mass of approximately 30% found in the patients in the present study after 12 months of EPO therapy shows that such an improvement can be achieved solely by correction of the anaemia. The decrease in cardiothoracic ratio seen on chest radiology is also likely to have been due to the decreased LV mass, plus or minus a reduction in ventricular volumes. Whilst London et al. [170] found a decrease in left ventricular end-diastolic diameter, the results of the present study, though showing a downward trend, failed to reach statistical significance. Unlike the other measurements in this study where no improvement occurred after the first few months of treatment, LV mass showed a progressive decline with time over the 12 months of observation, emphasising the benefits of maintaining a higher haematocrit for an extended period. This confirms and extends single end-point studies in this [171]

and another group [170] of haemodialysis patients, both of which showed a similar reduction in LV mass index. Whether this change will be associated with improved survival of dialysis patients will require long-term studies, but there is circumstantial evidence to suggest that this might be the case [148]. A number of other echocardiographic parameters have also been shown to improve after EPO therapy [172], although in the same study no changes were found in the thickness of the interventricular septum or the left ventricular posterior wall, possibly due to the rather short follow-up.

In conclusion, EPO treatment of uraemic anaemia is associated with improvements in cardiac function as judged by exercise capacity and oxygen consumption as well as a decrease in myocardial ischaemia and left ventricular mass. These benefits are maintained over at least 12 months of therapy.

Table 6 Exercise ECG S-T segment changes recorded in 10 haemodialysis patients before, and after 2, 4, 8, and 12 months of EPO therapy

Initials	Site	Pre-EPO	2 mths	4 mths	8 mths	12 mths
SW	L	2.0	-			
	I	-	-			
HL	L	1.0	-	-	-	-
	I	1.2	-	1.2	-	-
BB	L	1.0	-	-	1.4	-
	I	1.2	1.4	-	-	-
RP	L	2.0	-	-	-	-
	I	-	-	-	-	-
EE	L	1.0	-	-	-	-
	I	-	-	-	-	-
SR	L	-	-	-	-	-
	I	-	-	-	-	-
AF	L	1.0	-	-	-	-
	I	1.0	-	-	-	-
CL	L	2.2	-	-	-	-
	I	1.2	-	-	-	-
AG	L	3.0	-	-	-	-
	I	1.0	-	-	-	-
YJ	L	-	-	-	-	-
	I	-	-	-	-	-

Results shown are the shifts in S-T segments of ≥1 mm (0.1 mV) in leads V_5 (lateral (L) aspect of heart) and aV_f (inferior (I) aspect of heart). There were no significant changes in lead V_1 (anterior aspect of heart) in any of the patients during exercise.

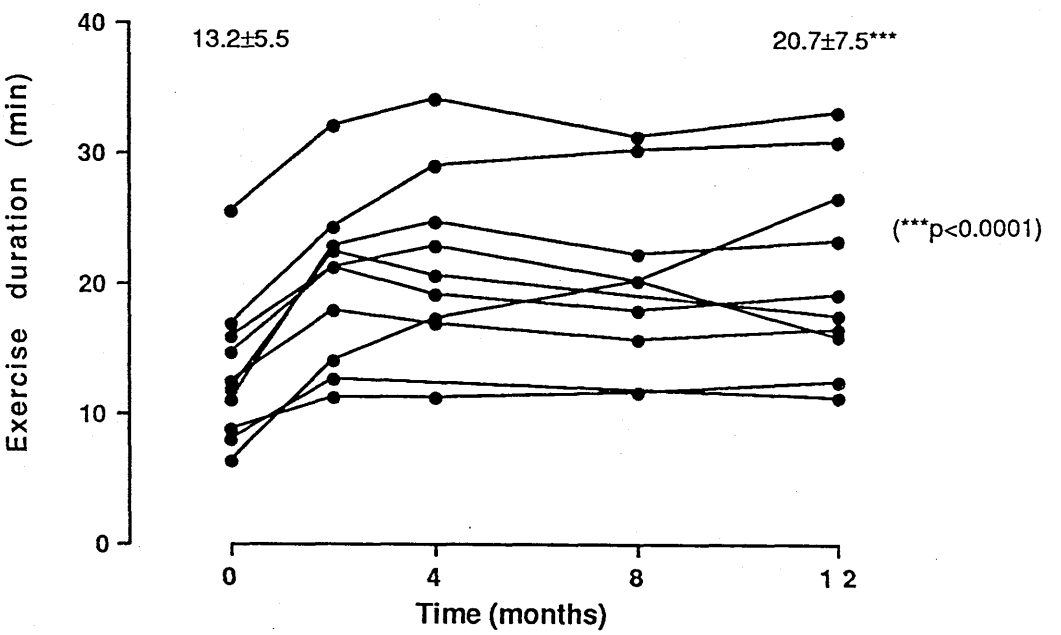


Fig. 21 Effect of EPO treatment on exercise duration during treadmill testing in 10 haemodialysis patients.

Data also given as mean ± SD for the group as a whole.

***p<0.0001.

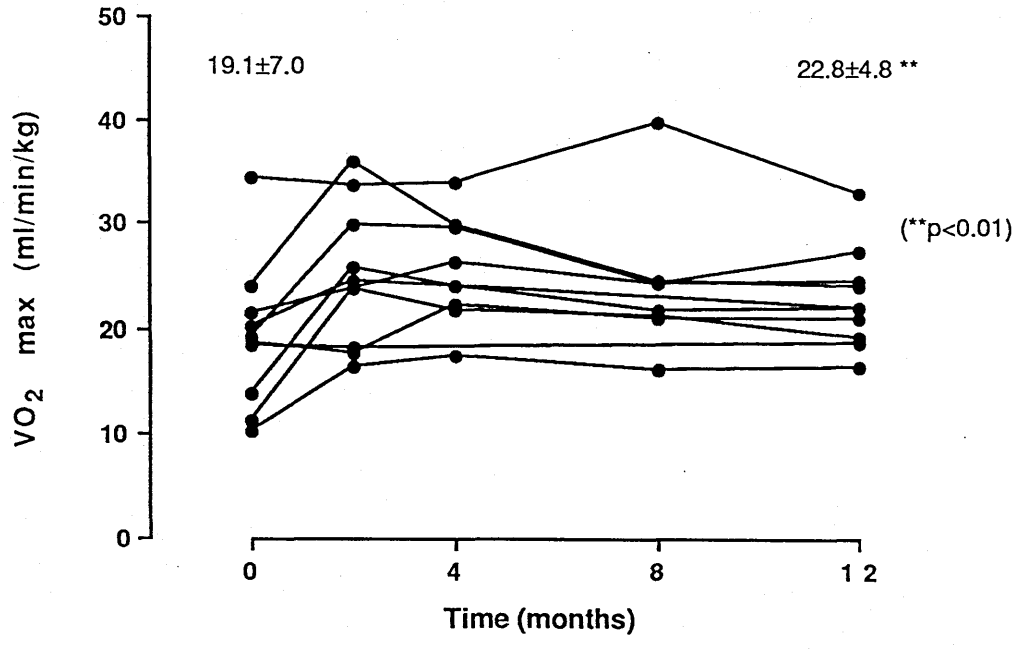


Fig. 22 Effect of EPO treatment on maximal oxygen consumption (VO_2 max) during treadmill testing in 10 haemodialysis patients.
Data also given as mean ± SD for the group as a whole.
**p<0.01.

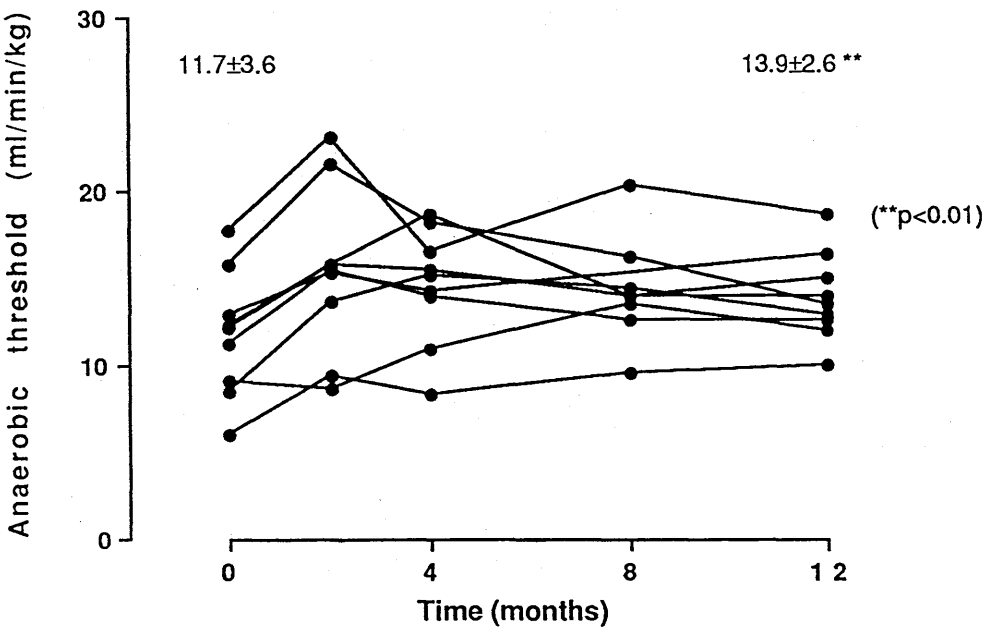


Fig. 23 Effect of EPO treatment on anaerobic threshold during treadmill testing in 9 haemodialysis patients.

Data also given as mean ± SD for the group as a whole.

**p<0.01.

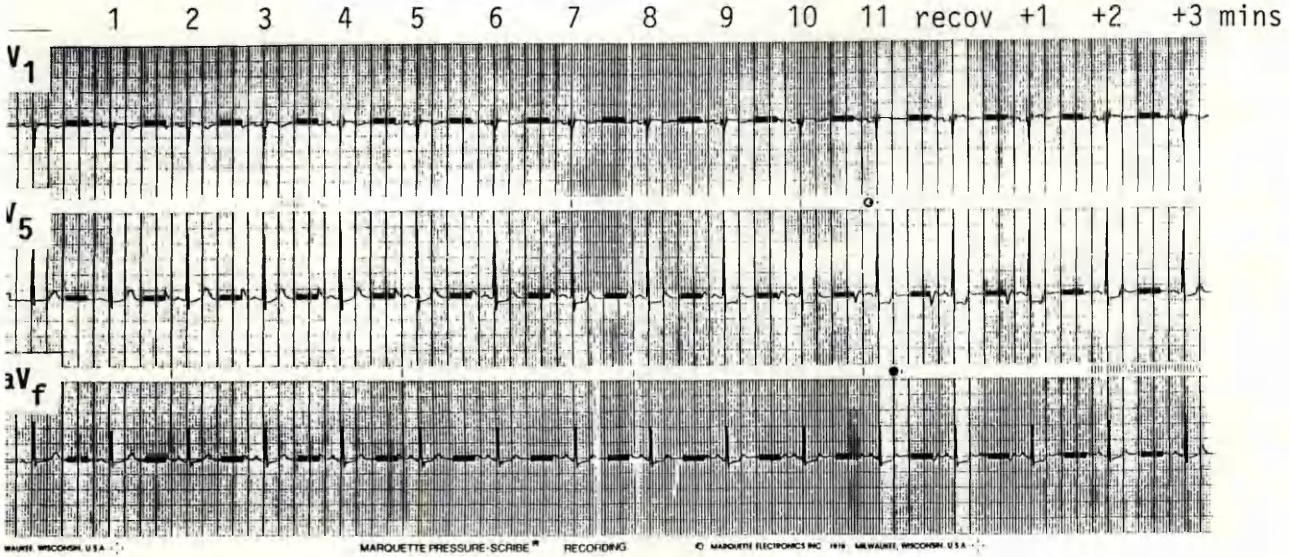
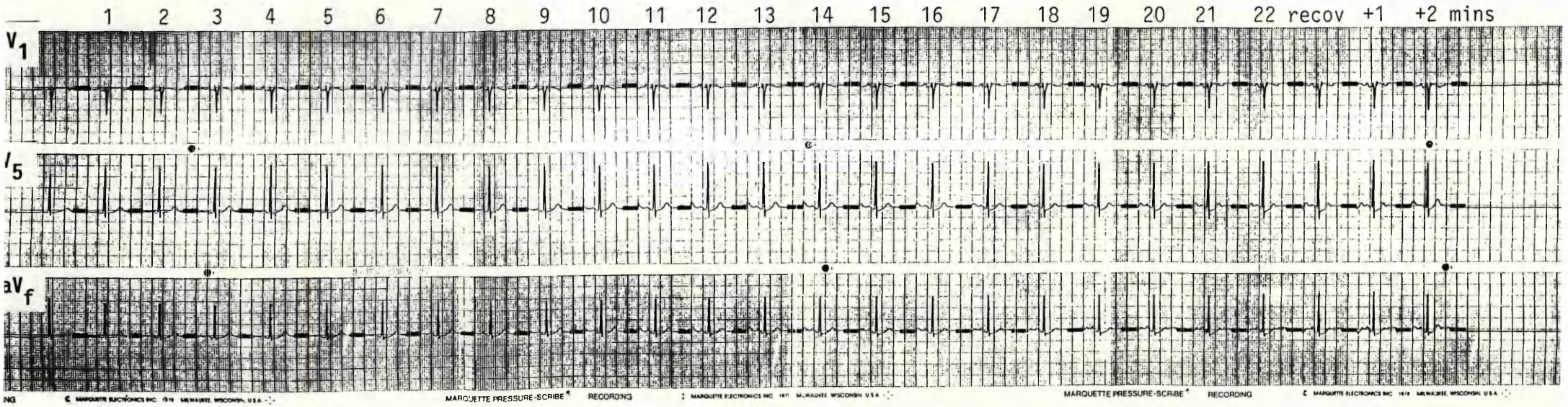


Fig. 24

Exercise ECG recorded at minute intervals during treadmill testing in a 52-year-old male haemodialysis patient treated with EPO. Note S-T segment depression of 2.2 mm in lead V_5 and 1.2 mm in lead aV_f after 11 minutes of exercise before EPO therapy was started. No ischaemic changes are present on repeat exercise ECG after 2 months of treatment despite a doubling of the exercise duration to 22 minutes.

After 2 months



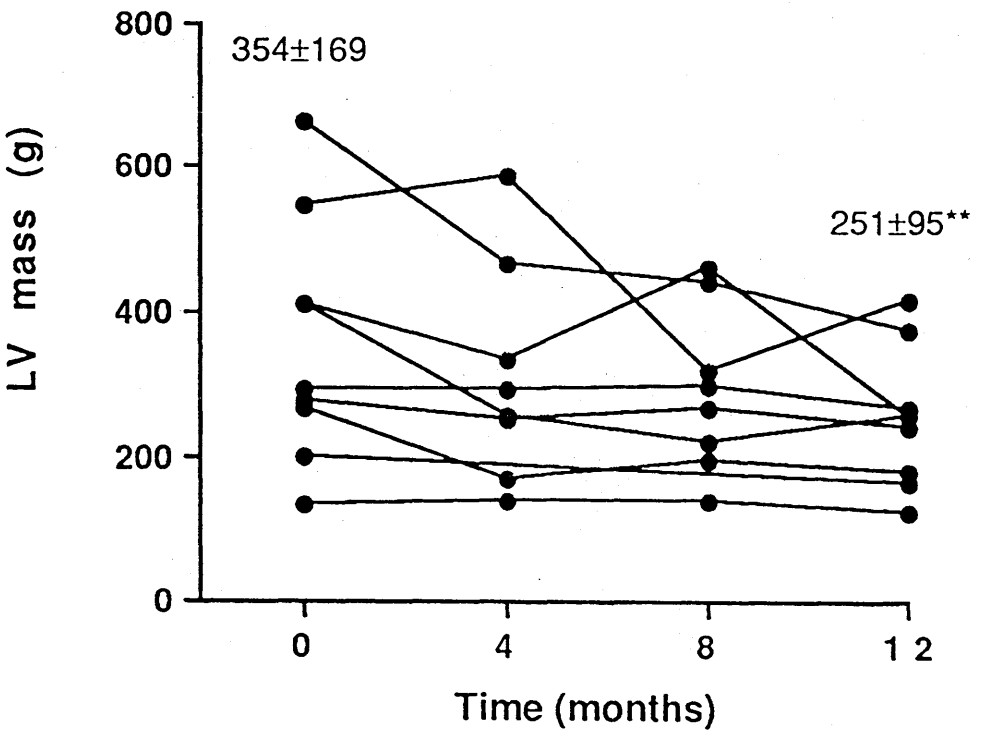


Fig. 25 Effect of EPO treatment on left ventricular (LV) mass in 9 haemodialysis patients.

Data also given as mean ± SD for the group as a whole.

**p<0.01.

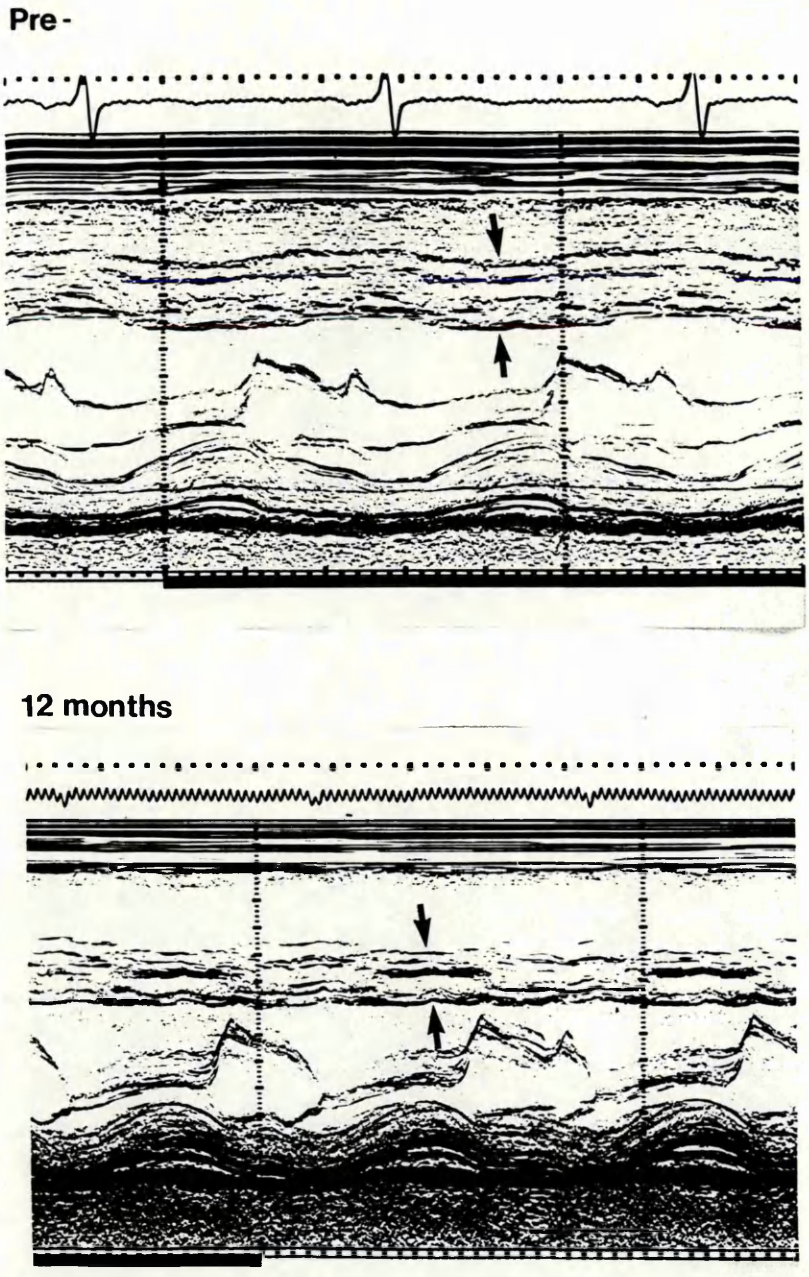


Fig. 26 Echocardiographic recording before and after 12 months of EPO therapy in a 26-year-old male haemodialysis patient.

Note reduction in thickness of inter-ventricular septum (arrows).

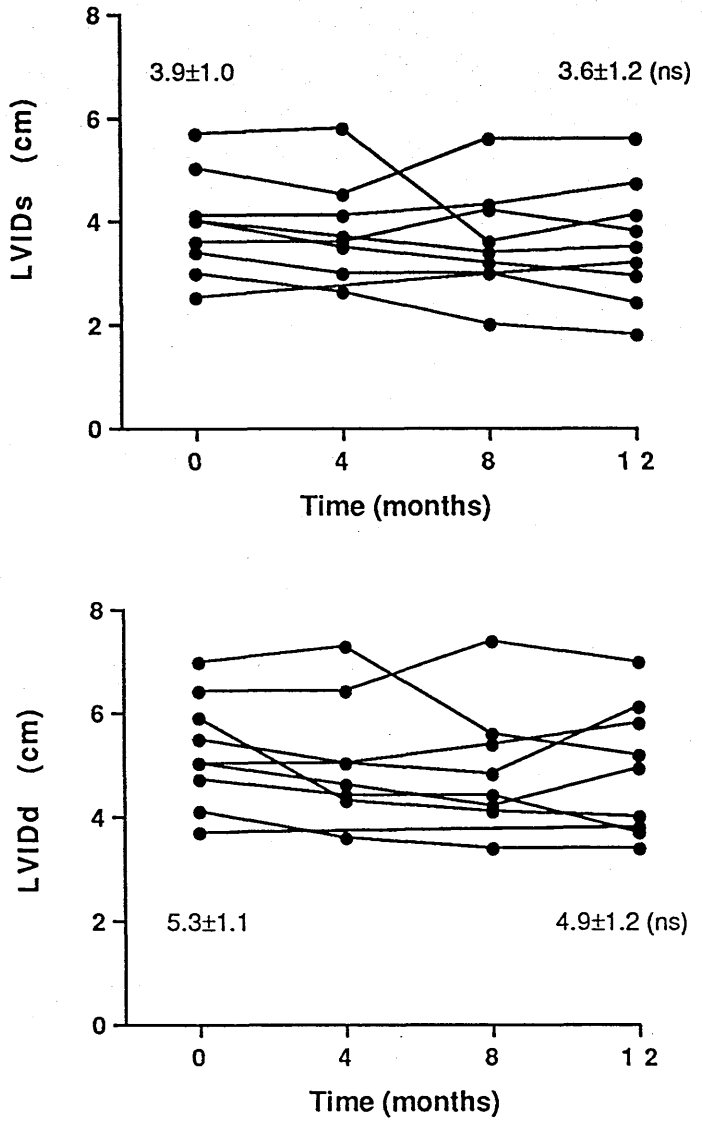


Fig. 27 Effect of EPO treatment on left ventricular internal dimensions in systole (LVIDs; upper panel) and diastole (LVIDd; lower panel) in 9 haemodialysis patients.

Data also given as means ± SD for the group as a whole.

ns = not significant.

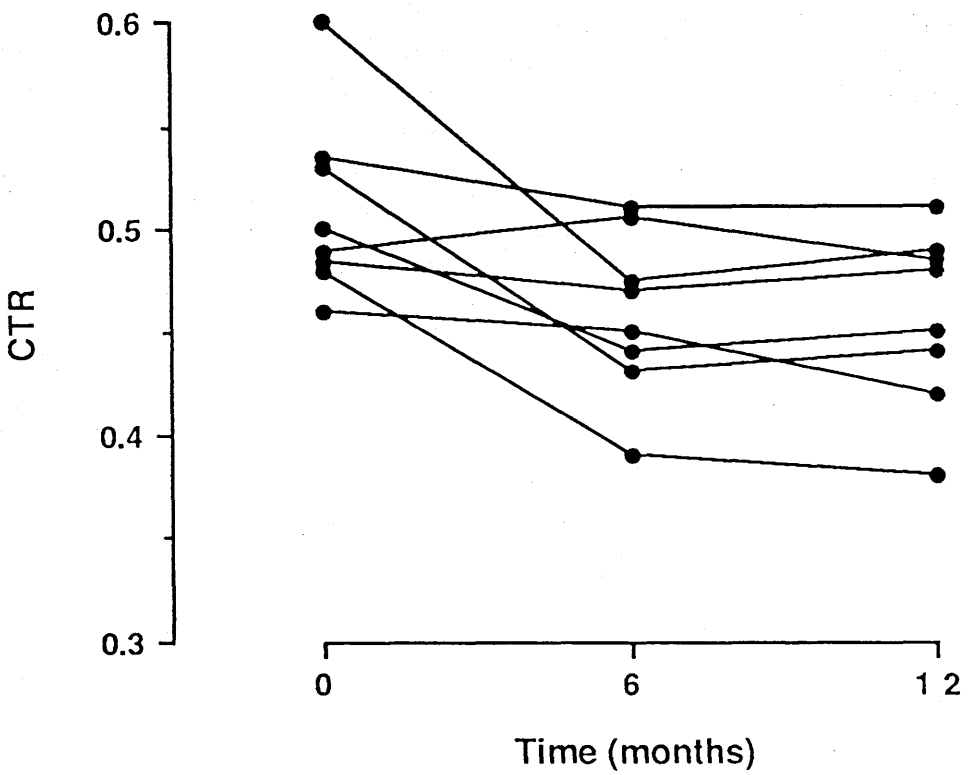


Fig. 28 Effect of EPO treatment on cardio-thoracic ratio (CTR) on a chest X-ray in 8 haemodialysis patients.

Data also given as mean ± SD for the group as a whole.

***p<0.005.

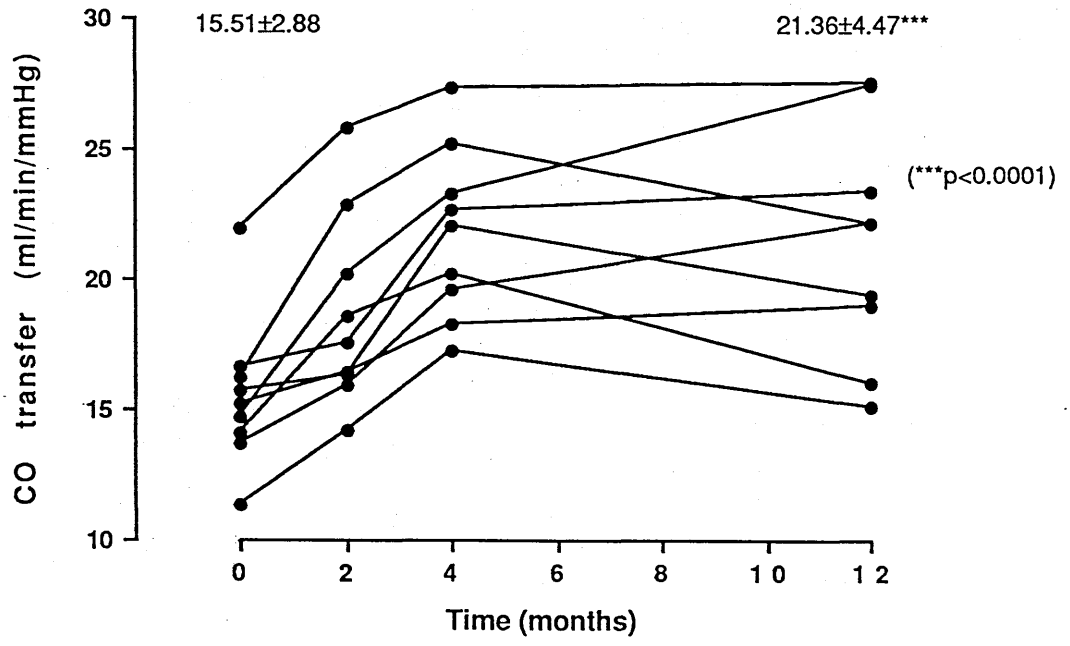


Fig. 29 Effect of EPO treatment on carbon monoxide (CO) transfer factor in 9 haemodialysis patients.

Data also given as mean ± SD for the group as a whole.

***p<0.0001.

CHAPTER 4

RHEOLOGICAL STUDIES DURING TREATMENT OF RENAL ANAEMIA WITH ERYTHROPOIETIN

INTRODUCTION

The previous two chapters have reported beneficial haematological and cardiorespiratory effects in anaemic dialysis patients treated with EPO. Many other studies have confirmed the efficacy of this new agent in the treatment of renal anaemia [1,2,3,4,126], and the patients themselves report dramatic improvements in well-being, physical capacity, and energy reserve [90,91,92,93]. Two major side-effects of this therapy, however, have emerged, namely hypertension (occasionally severe, resulting in encephalopathy) [1,2,3,4,90,91,92,93,173] and thrombosis of the arteriovenous fistula [90,92,93,174,175]. It has been suggested that the increase in blood viscosity associated with the rise in haematocrit plays a major role in the pathogenesis of these complications [160,173,176].

The purpose of the studies reported in this and the next chapter was to investigate potential aetiological factors in the development of hypertension and thrombotic events associated with EPO therapy. In this chapter is contained a detailed analysis of the rheological changes which occur following EPO treatment, and in Chapter 5 a study on the effect of EPO on a number of coagulation and haemostatic parameters is reported.

In addition to an increased quantity of circulating red cells [177,178], many other factors can potentially influence changes in whole blood viscosity. These include alterations in shear stresses secondary to changes in vascular dynamics, and changes in red cell aggregation and deformability [178] (which in turn are affected by the mean red cell volume (MCV), the mean red cell haemoglobin concentration (MCHC) [179], the presence of red cell inclusions, and alterations in the shape of the red cells [180]). Changes in plasma constituents such as fibrinogen [178], and in the characteristics of the white cells and platelets, will also have an effect. Patients with chronic renal failure not infrequently have abnormalities in one or more of these parameters, and the effect of EPO in this context has not been fully evaluated.

The present study outlines a number of rheological investigations in 30 dialysis patients treated with EPO with a view to characterising the associated changes in blood viscosity. Whole blood, plasma, and serum viscosities, and plasma fibrinogen levels,

were measured in 10 haemodialysis patients during EPO therapy. Red cell deformability, MCV, MCHC, and reticulocyte count were monitored in a further two groups of dialysis patients (10 haemodialysis, 10 CAPD) receiving EPO.

PATIENTS AND METHODS

Patients

Thirty patients were studied. All were severely anaemic (haemoglobin <8.0 g/dl) with normal serum ferritin, vitamin B_{12}, and folate levels, and no obvious cause for anaemia other than their renal failure. They were divided into three groups:-

Group 1: 10 haemodialysis patients treated initially with 240 U/kg/week of EPO (Boehringer Mannheim GmbH, Germany) given intravenously in 2 separate doses at the end of dialysis. These comprised 6 males and 4 females; mean age 43±16 (SD) years; mean duration on haemodialysis 4.4 years (range 0.6-14.7 years). Three of the patients were transfusion-dependent prior to receiving EPO. The cause of their renal disease was chronic glomerulonephritis (4 patients), chronic pyelonephritis/interstitial nephritis (2 patients), reflux nephropathy (2 patients), hypertensive nephropathy (1 patient), and congenital renal hypoplasia (1 patient). Their mean pre-treatment haemoglobin concentration was 6.4±0.6 g/dl.

Group 2: 10 CAPD patients treated initially with 120 U/kg/week of EPO administered subcutaneously in 2 separate doses. These consisted of 3 males and 7 females; mean age 54±18 years; mean duration on CAPD 4.1 years (range 1.7-8.2 years). None of the patients was transfusion-dependent prior to receiving EPO. The cause of their renal disease was chronic glomerulonephritis (2 patients), chronic pyelonephritis/interstitial nephritis (2 patients), hypertensive nephropathy (1 patient), obstructive uropathy (1 patient), post-partum renal failure (1 patient), and chronic renal failure of unknown aetiology (3 patients). The mean pre-treatment haemoglobin concentration for this group was 6.1±0.9 g/dl.

Group 3: 10 haemodialysis patients treated initially with 120 U/kg/week of EPO administered subcutaneously in 2 separate doses. These comprised 5 males and 5 females; mean age 37±19 years; mean duration on haemodialysis 2.7 years (range 0.3-6.6 years). Two of the patients were transfusion-dependent prior to receiving EPO. The cause of their renal disease was IgA nephropathy (1 patient), chronic pyelonephritis/interstitial nephritis (1 patient), reflux nephropathy (3 patients), obstructive uropathy (2 patients), polycystic kidneys (1 patient), and chronic renal failure of undetermined cause (2 patients). Their mean pre-treatment haemoglobin concentration was 6.8±0.7 g/dl.

In addition, 2 groups of normal, healthy volunteers drawn from the hospital staff were recruited to provide control data. The first of these (Group A) comprised 27 subjects (13 males, 14 females; mean age 32±7 years; mean haemoglobin concentration 14.6±1.3 g/dl) in whom whole blood and plasma viscosity was assessed. The second group (Group B) consisted of 30 subjects (15 males, 15 females; mean age 34±7 years; mean haemoglobin concentration 14.5±1.3 g/dl) in whom red cell deformability was measured.

Methods

All blood samples for rheological studies were taken with minimal stasis from an antecubital vein into bottles coated with EDTA K_2 (1.5 mg per ml blood), and tested within 3 hours.

Full blood count indices including haemoglobin concentration, haematocrit, MCV, and MCHC were measured weekly on a Technicon (Basingstoke, UK) H1 automated blood cell analyser up to 4 months of treatment, and measured monthly thereafter. The reticulocyte count was estimated by a single observer from a blood film stained with brilliant cresyl blue by counting the number of reticulocytes in 500 red cells.

Whole blood viscosity was measured at 37°C in the Group A control subjects on one occasion, and in the 10 Group 1 patients before EPO was commenced and every subsequent two weeks up to 4 months of treatment, and again after 12 months of treatment. This was performed using a Haake (Karlsruhe, Germany) CV 100 rotational viscometer. Measurements were made at shear rates of 3, 30, and 300 s^{-1} on whole blood, semi-packed cells, and packed cells (the latter two preparations made by decanting increasing amounts of plasma after centrifugation at 2,000 g for 10 minutes, and then thoroughly resuspending the cellular components). Plasma viscosity was measured at 50 s^{-1} (this having previously been found to give optimal sensitivity for plasma) on the Haake CV 100 system. This value for plasma viscosity was used as the viscosity at zero haemoglobin concentration for all shear rates, assuming that the plasma was behaving as a Newtonian fluid (i.e. viscosity independent of shear rate). Results are expressed as means ± SD with viscosity measured in mPa sec.

The natural logarithm of the viscosity was plotted against the haemoglobin concentration for the 3 shear rates, and the slopes derived for each set of measurements [181] (Fig. 30). The slopes of these lines (sl 3 s^{-1}, sl 30 s^{-1}, and sl 300 s^{-1} respectively) are sensitive to two major factors. These are (i) the ability of the red cells to disaggregate and to streamline under the effect of shear (this reflects the shear rate, the forces tending to hold the red cells together, and, particularly at high shear, the individual deformability of the red cells); and (ii) the plasma viscosity, as it is both a fixed point for the line, and its contribution to the whole blood viscosity decreases with increasing haemoglobin concentration. The natural logarithm of the viscosity at a calculated haemoglobin concentration of 15 g/dl (ln V) was also plotted against the natural logarithm of the respective shear rates (ln sh) to yield the gradient, ln V/ln sh. This value reflects the increasing effect of shear forces in being able to disaggregate and streamline the red cells in the direction of flow.

Plasma (EDTA-anticoagulated) and serum viscosities were determined at 37°C using a Luckham (Burgess Hill, UK) capillary viscometer. This system allows a greater precision than the Haake CV 100 rotational viscometer for plasma and serum, allowing more precise comparison of change in these parameters with time. It is, however, not suitable for measuring whole blood viscosity. Results are expressed as means ± SD, with values quoted in mPa sec.

Plasma fibrinogen was measured in aliquots of citrated plasma using an assay based on the method of Clauss [182]. Results are expressed in mg/dl (normal range 200-500 mg/dl).

Red cell deformability was determined in 30 control subjects (Group B), and in patient groups 2 and 3 before EPO was started, every subsequent two weeks up to 4 months of treatment, and thereafter monthly up to 9 months of therapy. This was assessed by filtering a red cell suspension through a 5 μm filter [183,184]. Before filtration, white cells were removed by adherence in a 5 cm cellulose column (50% α-cellulose; 50% microcrystalline cellulose, Sigma Chemicals). Approximately 3-5 ml of EDTA-anticoagulated whole blood was loaded onto the column and flushed through with phosphate-buffered saline (PBS; pH 7.4). Absence of white cells in the effluent was confirmed by analysis on a Technicon (Basingstoke, UK) H1 analyser. The resulting red cell suspension was then further diluted in PBS to achieve a final concentration of around $5x10^{11}$ red cells per litre. The volume of red cell suspension passing through a 5 μm Hemafil polycarbonate membrane (Nuclepore Corp., Pleasanton, California, USA) in 10 seconds under a positive pressure of 5 cm of water was then calculated by microcomputer, and compared to the volume of PBS previously passed through the same filter. The mean transit time (in msec) per red cell was calculated by analysis of the flow curves by the microcomputer controlling the process. Full details of the methodology and analysis have been published [183,184].

Analysis of results

Analysis of variance and the two-tailed paired t test were used to compare results at different time intervals. Unpaired t tests were used to analyse the differences in results for the control groups versus the dialysis patients prior to EPO therapy. Values are expressed throughout as means ± SD for each group.

RESULTS

Haemoglobin response. In all 3 groups of patients the haemoglobin concentration rose over the first 4 months of therapy to reach the target range of 10-12 g/dl (see Chapter 2, Fig. 9). There was no significant difference between the two subcutaneously-treated groups and the intravenously-treated group of patients. With adjustments in the dose of EPO, the haemoglobin concentration was maintained within the target range thereafter. There were no significant changes in the white cell or platelet counts after EPO (data not shown).

Whole blood viscosity. Whole blood viscosity increased over the first 4 months of EPO treatment at all 3 shear rates (Fig. 31). As expected, the differences were more marked the lower the shear rate (3.16±0.62 to 8.08±1.31 mPa sec (3 s^{-1}); 2.12±0.29 to 3.83±0.53 mPa sec (30 s^{-1}); and 1.88±0.29 to 2.84±0.22 mPa sec (300 s^{-1}). These results represent a 2.6-, 1.8-, and 1.5-fold rise in WBV over the first 4 months for the 3, 30, and 300 s^{-1} shear rates respectively. Repeat measurements at 12 months showed no significant difference from those obtained at 4 months (Table 7, Fig. 31). When the WBV data are plotted against the values of haemoglobin concentration, the relationship can be seen to follow an exponential function (Fig. 32) where r = 0.89 at 3 s^{-1}, 0.96 at 30 s^{-1}, and 0.95 at 300 s^{-1}. Thus, for a linear increase in the haemoglobin concentration, there was a disproportionate increase in the WBV, most marked the lower the shear rate. Nevertheless, the maximum values obtained in this group of patients were still considerably lower than in the normal healthy volunteers (Table 7). In the patient group, there were no significant changes in

the slopes of the shear rates (sl 3 s^{-1}, sl 30 s^{-1}, and sl 300 s^{-1}) measured over the first 4 months of therapy, with most values being similar to those in the control group (Table 7). The one exception to this was that the dialysis patients before and after EPO had slightly greater slopes for the 3 s^{-1} shear rate than did the normal volunteers. When the WBV 300 s^{-1} values in the patients were corrected to standard haemoglobin concentrations of both 10 and 15 g/dl, they were comparable to the values obtained from the healthy volunteer group, except at 12 months when they were slightly higher in the patient group (Table 7). This was due to the combination of a marginally higher PV and sl 300 s^{-1} at 12 months compared to baseline, and the difference becoming exaggerated due to the logarithmic relationship. The gradient ln V/ln sh, however, was consistently greater in the EPO-treated patients than in the control subjects at all time-points measured (Table 7); this is a consequence of the greater slopes for the 3 s^{-1} shear rate in the dialysis patients. This difference between the control and patient groups is likely to be due, at least in part, to the direct effect of the slightly higher plasma viscosity increasing red cell to red cell interactions.

Plasma/serum viscosity. There was no significant difference between the mean plasma viscosity for the haemodialysis patients before receiving EPO and that for the control group of healthy volunteers, both values being within the normal range. No significant changes in either plasma or serum viscosity were found during the period of study (Fig. 31).

Plasma fibrinogen. Consistent with the lack of change in the plasma viscosity results with EPO, no alterations were found in the levels of plasma fibrinogen during the first 4 months of therapy, the values obtained remaining within the normal range of 200-500 mg/dl (Table 7).

Red cell deformability. Although there was some variation in the red cell transit time in both groups of patients during the first 9 months of EPO therapy, no

significant changes in this parameter were found compared to baseline measurements (Fig. 33). Likewise there were no significant differences between the results for the haemodialysis patients versus the CAPD patients, nor the EPO-treated patients as a whole versus the control group of healthy volunteers (Fig. 33).

MCV/MCHC/reticulocyte count. Although in some patients an increase in MCV was found in the early stages of therapy, particularly if there was a marked reticulocytosis, as a group there were no significant changes in this parameter during the period of study (Table 8). One patient, however, had a persistently high MCV throughout (100-108 fl), and it was of note that she generally had slower transit times than the other patients in the group. There was a slight but progressive decrease in the MCHC in both Group 2 and Group 3 patients, reaching significant levels after about 8-10 weeks (Table 8). This was thought to be due to the development of early functional iron deficiency [123,124,125]. As expected, there was a significant increase in the reticulocyte count following EPO therapy of around 2-3 times the pre-treatment value (Table 8). This, however, was not manifested in any detectable change in red cell deformability.

DISCUSSION

This study represents the first detailed sequential analysis of the rheological changes occurring during the correction of renal anaemia with EPO therapy [185]. The data presented here confirm the expected increase in whole blood viscosity associated with the EPO-induced rise in haemoglobin concentration [160,173]. It is at the lower shear rates that the exponential increase in blood viscosity with haemoglobin concentration is most apparent, due to an increase in cell to cell interaction and a decrease in the streamlining of the red cells seen at high rates of shear. High plasma viscosity and elevated red cell aggregation can critically limit blood flow in the microcirculation, while the flow in large blood vessels predominantly depends on the haematocrit [178]. The increase in WBV is appropriate for the rise in haemoglobin

concentration at all stages of treatment, suggesting that it is occurring solely as a result of this increase in circulating red cell mass. Consistent with this is the lack of any change in the plasma viscosity, or in the rheology of the red cells themselves as assessed by their deformability.

Schaefer et al. [173] also reported increases in WBV at low (2 s^{-1}) and high (100 s^{-1}) shear rates which did not reach the values seen in healthy subjects. Unlike the present study these workers determined WBV in native blood only, and not in samples with an adjusted haematocrit. In contrast to the present study, these authors reported elevated plasma viscosity measurements and fibrinogen levels in their haemodialysis patients prior to EPO [173]. As with the results reported in this study, however, Schaefer et al. [173] noted a slight but insignificant rise in these parameters after 16 weeks of therapy. Using a controlled stress rheometer, Brunner et al. [186] demonstrated a rise in the apparent WBV from 2.52±0.24 to 2.86±0.39 mPa sec associated with an increase in the haematocrit from 0.25 before EPO to 0.36 after 12 weeks' therapy. Determination of apparent WBV curves against different haematocrits, however, showed that the increase in WBV after 12 weeks was less than predicted from the rise in haematocrit and the initial WBV curve. This contrasts with the findings in the present study which indicate that the rise in WBV on repeated measurement up to 16 weeks correlated very well with what was anticipated from the initial WBV curve. The less-than-expected increase in WBV in the study by Brunner et al. [186] was attributed to a transient reduction in erythrocyte aggregation seen after correction of the anaemia by EPO. Mayer et al. [187], however, obtained data consistent with the results reported here in that, when the rise in WBV was corrected for the increase in haematocrit, no change in blood viscosity was evident.

Several groups of workers have reported abnormalities of red cell deformability in undialysed patients with end-stage renal failure [188,189,190]. Regular dialysis treatment, however, restores the red cell deformability to normal [190], consistent with the findings in the present study. Although there have been reports of impaired red cell filterability in patients undergoing haemodialysis [189,191,192,193,194] or

CAPD [189], these studies were carried out using erythrocyte suspensions that were contaminated with leucocytes, to which filtration is extremely sensitive [183,184,195]. In the present study, absence of white cells in the preparation was confirmed prior to measurement of erythrocyte deformability. Red cell rheology is also affected by changes in the MCV, MCHC, and erythrocyte shape [179,180]; all three of these parameters may be abnormal in chronic renal failure.

Lerche et al. [196] studied a number of parameters of red cell rheology in haemodialysis patients before, and after 7 and 30 weeks of EPO therapy. These workers found significant reductions in the apparent membrane elastic shear modulus and capillary rigidometer entry time of red cells (assessed by micropipette aspiration techniques), but no change in the red cell aggregation index, Dublett time, or plasma viscosity. These techniques, however, assessed only relatively small numbers of erythrocytes (about 25-100 cells per sample). A recent study by Koppensteiner et al. [197] showed an increase in red cell aggregation in haemodialysis patients treated with EPO which was attributed to the increasing number of red cells. Their finding that at low shear rate the value exceeded that seen in the control subjects is consistent with the data in the present study showing differences in the sl 3 s^{-1} (see Table 7).

In summary, therefore, a rise in WBV has been universally found following an increase in haematocrit with EPO [173,176,186,187]. The results of the present study suggest that the progressive increase in WBV can be attributed solely to the increased haematocrit, rather than to any changes in erythrocyte rheology or plasma viscosity. The discrepancy between the findings documented here and the few reports of alterations in red cell deformability [196] or aggregation [186] may be accounted for by differences in the methodology used. If, however, changes in red cell rheology do occur with EPO therapy then it would appear that their contribution is too small to have a significant effect on WBV, of which haematocrit is the most important single determinant [177].

The clinical relevance of these findings remains unclear. Since blood viscosity, by mechanisms not completely understood, appears to determine vascular resistance

[178,198,199,200] and thereby blood pressure [201,202,203], it has been suggested that the rise in WBV plays a major role in the pathogenesis of hypertension in patients treated with EPO [160,173,176]. Nevertheless, as seen in the present study, the absolute values of WBV obtained at the target haemoglobin concentration are still considerably lower than those in normal individuals. Furthermore, Steffen et al. [204] found no correlation between the changes in WBV and the increases in blood pressure in 10 haemodialysis patients receiving EPO. Finally, Williams et al. [205] acutely raised the haematocrit, and hence the WBV, in 100 haemodialysis patients by transfusion, and found no significant increase in blood pressure. Similarly, there was no evidence of a pressor effect in 100 CAPD patients in whom a spontaneous rise in haemoglobin concentration was observed within the first 12 months of commencing dialysis [205].

The contribution of the EPO-induced rise in WBV to the increased risk of developing thrombotic side-effects, particularly of the arteriovenous fistula [90,92,93,174,175], is also uncertain. An increase in blood viscosity to supra-normal levels, however, is associated with frequent thrombotic events in patients with polycythaemia or Waldenstrom's macroglobulinaemia. Other factors, such as alterations in platelet function [206,207,208,209] or a reduction in protein C and protein S levels (see Chapter 5) [210] after EPO therapy may also contribute. On a more positive note, the increase in WBV associated with EPO may play a role in the reversal of erectile impotence reported in male haemodialysis patients after EPO therapy [211].

Table 7 Rheological data obtained from normal volunteers and haemodialysis patients treated with erythropoietin during the first 12 months of therapy

		PATIENTS - TIME (weeks) AFTER EPO COMMENCED									
	NORMAL VOLUNTEERS	0	2	4	6	8	10	12	14	16	52
Hb (g/dl)	14.6 (1.3)	6.4 (0.6)¥	7.4 (1.0)	8.5 (1.2)	9.2 (1.5)	10.1 (1.7)	10.7 (1.1)	10.9 (0.9)	11.7 (0.8)	11.5 (0.7)¥	10.8 (0.7)¥
Hct	-	0.194 (0.02)	0.225 (0.035)	0.250 (0.04)	0.266 (0.038)	0.286 (0.052)	0.307 (0.039)	0.317 (0.045)	0.340 (0.063)	0.339 (0.029)	0.326 (0.013)
WBV 3 s^{-1}	10.08 (1.92)	3.16 (0.62)¥	4.46 (0.99)	4.75 (0.96)	5.42 (0.87)	5.84 (1.07)	6.88 (1.03)	6.80 (0.88)	7.73 (1.25)	8.08 (1.31)§	7.37 (1.04)¥
WBV 30 s^{-1}	4.92 (0.68)	2.12 (0.29)¥	2.35 (0.32)	2.74 (0.36)	2.94 (0.44)	3.20 (0.57)	3.38 (0.42)	3.42 (0.40)	3.86 (0.49)	3.83 (0.53)¥	3.81 (0.30)¥
WBV 300 s^{-1}	3.42 (0.44)	1.88 (0.29)¥	2.03 (0.22)	2.28 (0.22)	2.41 (0.27)	2.43 (0.32)	2.61 (0.26)	2.62 (0.21)	2.84 (0.18)	2.84 (0.22)¥	2.81 (0.21)¥
sl 3 s^{-1}	0.145 (0.005)	0.154 (0.006)¥	0.155 (0.008)	0.151 (0.007)	0.154 (0.005)	0.152 (0.007)	0.155 (0.009)	0.151 (0.006)	0.155 (0.007)	0.154 (0.007)¥	0.154 (0.006)¥
sl 30 s^{-1}	0.102 (0.003)	0.104 (0.005)	0.106 (0.005)	0.106 (0.005)	0.106 (0.004)	0.103 (0.007)	0.104 (0.004)	0.103 (0.004)	0.104 (0.005)	0.103 (0.005)	0.106 (0.004)§
sl 300 s^{-1}	0.082 (0.003)	0.081 (0.004)	0.081 (0.004)	0.083 (0.004)	0.083 (0.003)	0.080 (0.004)	0.080 (0.004)	0.079 (0.002)	0.080 (0.003)	0.080 (0.003)	0.084 (0.003)
Cal Vis Hb 10	2.60 (0.11)	2.61 (0.11)	2.59 (0.19)	2.69 (0.13)	2.66 (0.05)	2.59 (0.09)	2.62 (0.14)	2.58 (0.11)	2.65 (0.09)	2.67 (0.13)	2.76 (0.19)¥
Cal Vis Hb 15	3.92 (0.19)	3.91 (0.23)	3.89 (0.35)	4.08 (0.26)	4.02 (0.12)	3.86 (0.17)	3.91 (0.26)	3.72 (0.21)	3.95 (0.15)	3.98 (0.22)	4.19 (0.27)¥
ln V / ln sh	-0.218 (0.016)	-0.239 (0.014)¥	-0.259 (0.021)	-0.232 (0.021)	-0.247 (0.015)	-0.246 (0.024)	-0.254 (0.029)	-0.241 (0.026)	-0.248 (0.028)	-0.256 (0.014)¥	-0.243 (0.011)¥
PV	1.19 (0.05)	1.21 (0.04)	1.20 (0.06)	1.23 (0.05)	1.22 (0.03)	1.24 (0.05)	1.22 (0.05)	1.22 (0.05)	1.23 (0.05)¥	1.26 (0.05)¥	1.26 (0.11)*
SV	not measured	1.10 (0.06)	1.09 (0.03)	1.11 (0.03)	1.11 (0.04)	1.12 (0.04)	1.09 (0.05)	1.10 (0.04)	1.11 (0.02)	1.13 (0.04)	1.14 (0.06)
Fibrinogen (mg/dl)	not measured	276 (44)	284 (71)	326 (83)	270 (68)	296 (52)	293 (38)	304 (65)	284 (78)	298 (72)	not measured

*$p<0.05$, §$p<0.01$, ¥$p<0.005$ comparing patient results at 0, 16, and 52 weeks with those for normal volunteers. Results expressed as means (SD).

WBV - whole blood viscosity measured at 3 s^{-1}, 30 s^{-1}, and 300 s^{-1} shear rates.

sl - slopes of the lines for the relationship between the haemoglobin concentration and the natural logarithm of the viscosity for the 3 s^{-1}, 30 s^{-1}, and 300 s^{-1} shear rates.

Cal Vis Hb 10,15 - calculated whole blood viscosity at 300 s^{-1} shear rate corrected to standard haemoglobin concentrations of 10 and 15 g/dl.

ln V / ln sh - slope of the line for the relationship between the natural logarithm of the viscosity and the natural logarithm of the shear rate.

PV - plasma viscosity.

SV - serum viscosity.

Table 8 MCV, MCHC, and reticulocyte results in 2 groups of dialysis patients receiving EPO

	MCV (fl)		MCHC (g/dl)		Reticulocytes ($x10^9$/l)	
Time (wk)	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3
0	89.5 (5.7)	91.7 (8.7)	33.5 (0.6)	33.0 (1.8)	67.4 (29.6)	38.4 (8.3)
2	90.2 (4.7)	92.9 (8.4)	32.8 (0.7)	32.8 (1.3)	106.2 (48.0)	116.9 (52.2) ***
4	89.3 (5.4)	93.5 (8.9)	32.9 (1.1)	32.5 (1.3)	116.0 (53.5) **	99.5 (42.8) ***
6	89.2 (5.6)	93.5 (9.7)	33.5 (1.2)	32.5 (2.3)	133.8 (47.0) ***	88.0 (35.4) ***
8	89.6 (5.5)	94.6 (8.5)	32.7 (1.1)	32.6 (1.2)	131.4 (64.2) ***	75.6 (33.5) **
10	90.9 (6.1)	92.9 (8.3)	32.1 (0.8) ***	32.9 (1.5)	131.1 (76.1) *	70.2 (27.0) ***
12	90.0 (6.7)	92.1 (7.3)	32.7 (1.0) *	32.5 (1.3) *	136.1 (82.5) *	76.0 (60.4)
14	88.3 (7.1)	92.9 (7.8)	31.9 (1.3) **	31.5 (1.5) ***	130.4 (57.2) *	69.9 (20.9) ***
16	90.3 (7.6)	93.1 (9.9)	31.0 (1.2) ***	31.7 (1.0) ***	117.1 (41.7) *	90.4 (39.4) *

*p<0.05, **p<0.01, ***p<0.005 compared with baseline.

Results expressed as means (SD).

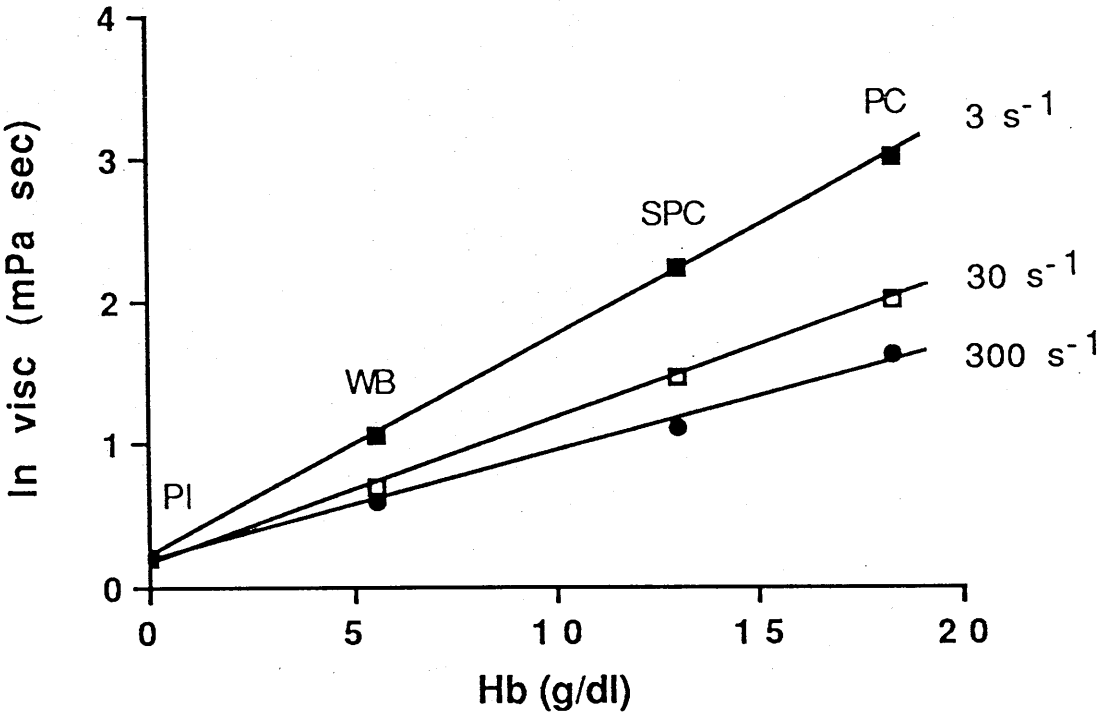


Fig. 30 Relationship between haemoglobin concentration and viscosity in one blood sample measured at 4 different haemoglobin concentrations and at 3 different shear rates (3 s^{-1}, 30 s^{-1}, and 300 s^{-1}).

Pl = plasma; WB = whole blood; SPC = semi-packed cells;

PC = packed cells; ln visc = natural logarithm of the viscosity.

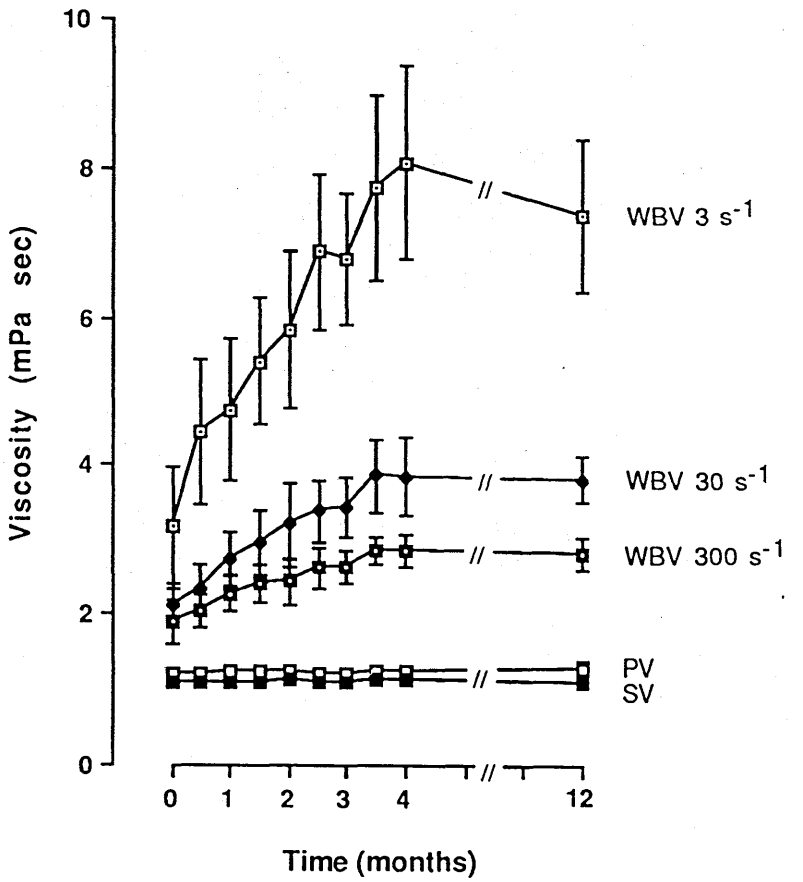


Fig. 31 Effect of EPO treatment on blood viscosity in 10 haemodialysis patients.

WBV = whole blood viscosity measured at shear rates of 3 s^{-1}, 30 s^{-1}, and 300 s^{-1}.

PV = plasma viscosity.

SV = serum viscosity.

Results expressed as means ± SD.

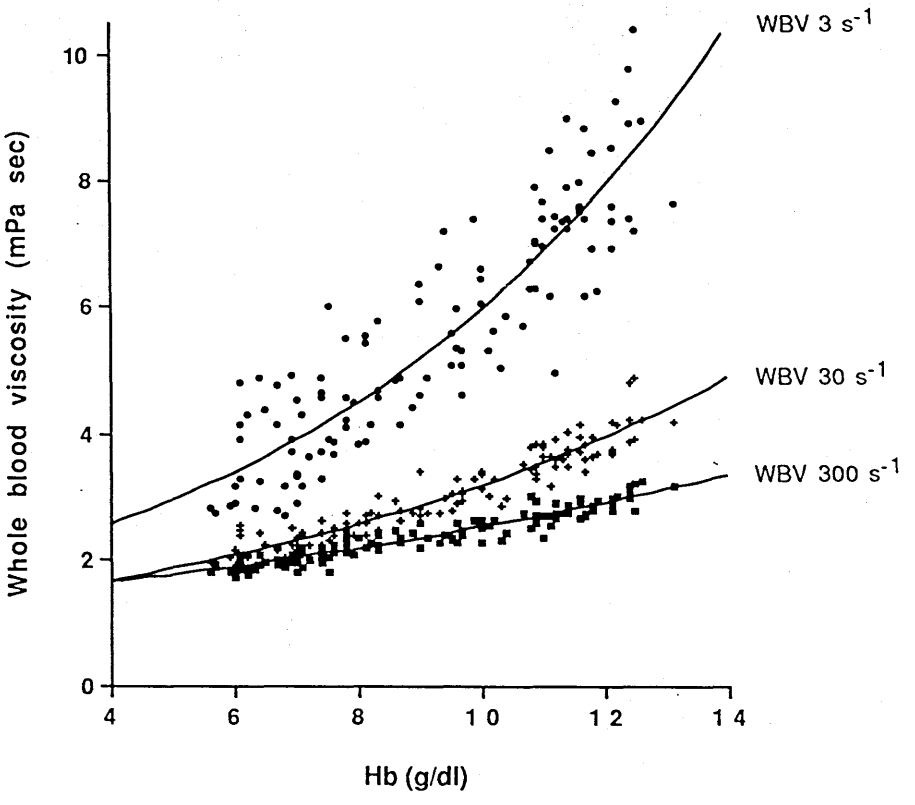


Fig. 32 Relationship between haemoglobin concentration and whole blood viscosity (WBV) measured at shear rates of 3 s^{-1}, 30 s^{-1}, and 300 s^{-1} in 10 haemodialysis patients receiving EPO over a 12-month period.

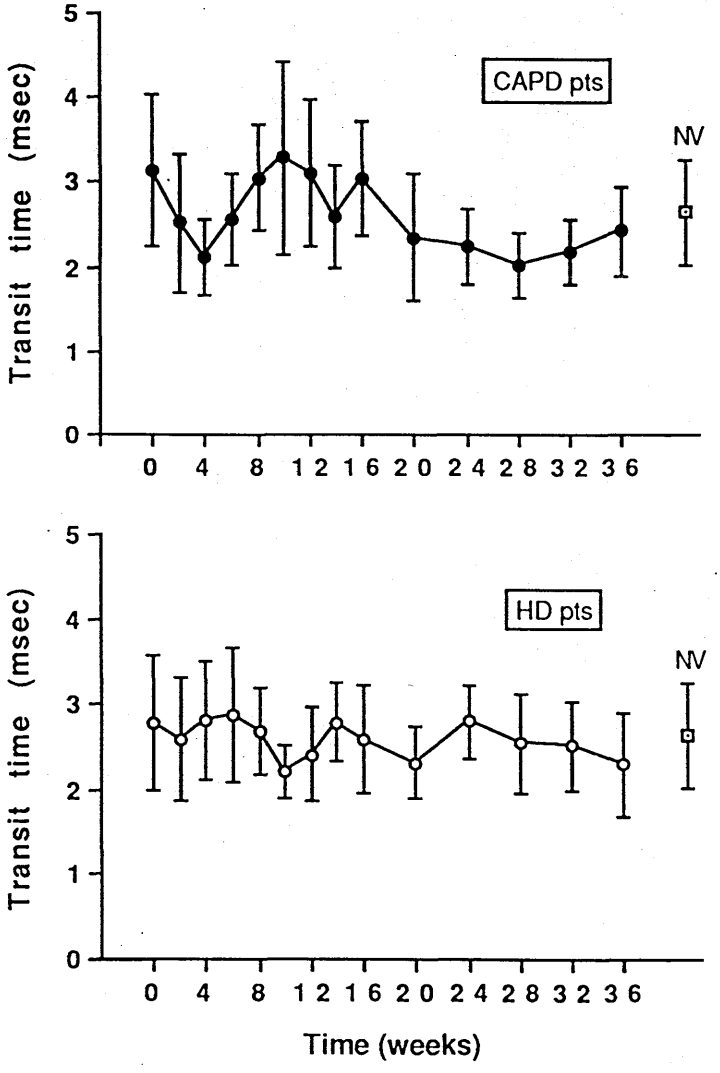


Fig. 33 Effect of EPO on red cell deformability in 2 groups of dialysis patients during the first 9 months of treatment. Results are expressed as means ± SD and compared with those for normal volunteers (NV) not on EPO.

Upper panel = CAPD patients.

Lower panel = haemodialysis patients.

CHAPTER 5

FISTULA BLOOD FLOW AND COAGULATION STUDIES DURING ERYTHROPOIETIN THERAPY

INTRODUCTION

Hypertension [1,2,3,4,90,91,92,93,173] and thrombosis of the arteriovenous fistula [90,92,93,174,175] are the two most worrying complications of EPO therapy in haemodialysis patients. Many of the early studies on EPO produced anecdotal reports of this latter problem developing in treated patients [90,92,93], and this was followed by results from a European multi-centre trial involving 150 haemodialysis patients which recorded an incidence of fistula thrombosis of 9-10% [174]. Clotting in the extra-corporeal circuit was found in a similar proportion of patients [174]. More recently a placebo-controlled multi-centre trial from Canada showed a significantly higher incidence of fistula thrombosis in patients treated with EPO (11 of 78 patients) compared to those given placebo (1 of 40 patients) [175]. Whether other thrombotic complications such as myocardial infarction and stroke occur more commonly than in untreated haemodialysis patients is as yet unknown.

Both the hypertension and the thrombotic complications do not appear to be related to the EPO *per se;* rather this has been attributed to the resultant increase in haematocrit and associated rise in blood viscosity (see Chapter 4) [160,173,185]. Other factors, however, may contribute to an enhanced risk of fistula thrombosis. Bleeding time is corrected or improved in the majority of patients on EPO therapy [185,206]. Changes in platelet function have also been reported [206,207,208,209]. Nevertheless, there have been no comprehensive studies published on the effect of EPO on the haemostatic system as a whole. This was the aim of the present study which monitored a variety of coagulation tests over the first few months of EPO therapy in 10 haemodialysis patients. In addition, fistula blood flow was measured by ultrasound using the Doppler technique.

PATIENTS

Group 1

Ten stable patients maintained on chronic haemodialysis were studied. Their demographic details including age, sex, aetiology of renal disease, duration on dialysis, and starting haemoglobin concentration are all as documented in Chapter 4 (Group 1 patients). One patient (34 years, female) had suffered 2 previous myocardial infarctions during the preceding 2 years and had subsequently undergone a right coronary angioplasty; another patient (75 years, male) had a past history of recurrent transient ischaemic attacks, and a further 2 patients had sustained previous thrombosis of their arteriovenous fistulae. Three of the patients were regular cigarette smokers. None of the patients received anticoagulant therapy except heparin during dialysis, and none had clinical or laboratory evidence of liver disease.

All patients were treated with EPO (Boehringer Mannheim GmbH, Germany) 120 U/kg given intravenously (IV) twice weekly at the end of dialysis. While receiving EPO therapy, 2 patients developed fistula thrombosis: in one (51 years, female), occurring at 13 weeks, blood flow was regained almost immediately following a sharp external blow to the fistula; in the other (22 years, male) surgical recanalisation was unsuccessful and a new fistula had to be constructed. No other thrombotic events were encountered during the first 12 months of EPO therapy.

Group 2

Protein C and protein S levels were monitored in a further 12 stable haemodialysis patients (6 males, 6 females; mean age = 37±18 years) who had been receiving regular dialysis for a mean of 2.5±2.3(SD) years. The cause of their renal disease was chronic pyelonephritis/reflux nephropathy (4 patients), interstitial nephritis (1 patient), lupus nephritis (1 patient), IgA nephropathy (1 patient), obstructive uropathy (1 patient), and polycystic kidney disease (1 patient), with three patients undiagnosed.

The mean haemoglobin concentration immediately before starting EPO ranged from 6.0 to 7.8 g/dl (mean 6.8 g/dl) and serum ferritin, B_{12}, and folate levels were as for the first group. The patients in this second group were treated with a starting dose of 60 U/kg of EPO (Boehringer Mannheim GmbH, Germany) administered subcutaneously (SC) twice weekly. None of these patients developed fistula clotting or any other thrombotic complications over the first 6 months of EPO therapy.

METHODS

All tests were carried out, and all samples taken, immediately prior to a dialysis session.

Haemoglobin concentration was measured weekly on a Technicon (Basingstoke, UK) H1 automated analyser up to 4 months of treatment, and measured monthly thereafter. Whole blood viscosity (WBV) was measured every 2 weeks up to 4 months of EPO therapy, and again at 12 months (results reported in Chapter 4). Bleeding time was determined every 2 weeks up to 4 months of EPO, and again at 12 months using the Simplate Bleeding Time Device on the non-fistula forearm.

The following tests of coagulation and/or haemostasis were determined on blood anticoagulated with sodium citrate 0.105 M (1 part citrate : 9 parts blood) every 2 weeks up to 4 months of EPO therapy: one-stage prothrombin time (OSPT), kaolin cephalin clotting time (KCCT), and whole blood clotting time (WBCT). All were measured using standard methods, and the prothrombin consumption index (PCI) calculated. Factor VII and factor VIII levels in plasma were measured by an optical density method using the Coag-A-Mate XC (Organon Teknika Ltd., Cambridge, UK) system. Plasma fibrinogen levels were determined using an assay based on the method of Clauss [182], again utilising the Coag-A-Mate XC system. Anti-thrombin III levels were measured using a chromogenic assay. Protein C and protein S (total and free) levels were measured by ELISA using commercially available kits (Boehringer Mannheim GmbH, Germany). Platelet aggregability was measured in platelet-rich plasma after dilution to a standard count of $120\text{-}150 \times 10^9$/l by means of impedance

aggregometry using ADP as the aggregating agent. A dose-response curve to increasing concentrations of ADP ($1x10^{-7}$ to $5x10^{-5}$ M) was constructed at each assessment, and the peak of aggregation was measured in millimetres.

Fistula blood flow was measured before EPO therapy, and after 1, 2, 3, 4, and 12 months of treatment by means of ultrasonography on a Toshiba Sonolayer SSA-100A system (Crawley, West Sussex, UK) using the Doppler technique. The average of three measurements was taken on each occasion, and results were expressed in litres per minute. The same operator performed the test throughout the study, and care was taken to use the same site for each assessment.

Analysis of variance and two-tailed paired t tests were used to analyse the statistical significance of any changes in the above parameters during EPO therapy.

RESULTS

Haemoglobin concentration

The rise in haemoglobin concentration (Hb) for both groups of patients is shown in Fig. 34. Both groups had fairly similar Hb responses despite the fact that the group treated IV received twice the dose of EPO compared to the group treated SC. Doses of EPO were adjusted to achieve or maintain a target Hb of 10-12 g/dl.

Coagulation/haemostasis tests

Seven of 10 patients had bleeding times greater than 30 minutes prior to EPO therapy (Table 9). This improved in 4 of these patients after 6 weeks, and by 16 weeks nine patients had a normal bleeding time (less than 10 minutes) and one patient had a borderline measurement. This improvement in bleeding time was still present at 12 months.

Platelet aggregability, one-stage prothrombin time, kaolin cephalin clotting time, whole blood clotting time, and prothrombin consumption index were all found to be within the normal range prior to EPO therapy, and no significant changes were noted during the first 4 months of treatment. Plasma fibrinogen levels were normal

throughout the first 4 months of EPO, consistent with the normal plasma viscosity results. Likewise, factor VII, factor VIII, and anti-thrombin III levels in plasma did not change with EPO therapy (Table 10).

Protein C and Protein S

Two baseline measurements of protein C obtained from group 1 patients were both within the normal range of 80-120% , the mean value immediately prior to treatment being 84.3±8.2% (Fig. 35). Levels of protein C remained unchanged during the first 2 months of EPO therapy but by 3 months there was a significant fall in protein C which by 4 months had reached levels (66.4±12.5%) thought to predispose to thrombosis (less than 70% [212,213]). After 8 months of EPO, however, protein C levels had returned to baseline values and this was maintained on re-testing at 12 months (85.4±17.0%). A similar fall in protein C levels was seen in the group 2 patients. Again, a significant reduction in protein C was found after 4 months of EPO therapy (Fig. 35).

Total protein S levels in group 1 patients began to fall after 1 month of EPO therapy having been in the upper end of the normal range on two occasions prior to EPO (mean value immediately prior to treatment: 124.1±24.3%) (Fig. 36). By 2 months a significant reduction was evident, and levels of protein S measured at 4 months (68.3±15.1%) were in the range believed to predispose to thrombotic complications (less than 70% [212,213]). As for protein C, protein S levels had returned to baseline by 8 and 12 months (118.1±32.8% and 107.9±23.7% respectively). A similar incremental fall in the concentrations of free protein S was found which paralleled the decrease in total protein S levels (61.5±25.6% pre-EPO; 37.1±10.6% at 4 months; 55.8±21.4% at 12 months) (Fig. 36). These results were confirmed in the second study on group 2 patients in which reductions in both total and free protein S were again observed (Fig. 36).

To elucidate whether this effect was directly related to EPO *per se* or to the correction of anaemia, a limited study was performed in an additional 5 anaemic

haemodialysis patients in whom protein C and protein S levels were measured before (2 measurements) and after (2 measurements) 4 units of packed cells. The mean Hb rose from 6.3±1.0 (SD) g/dl to 11.0±1.0 g/dl which is comparable to the increase seen in the EPO-treated patients. However, no alterations in protein C or protein S levels were observed with this acute correction of renal anaemia (protein C: 76.6±15.3% to 76.8±13.6%; total protein S: 104.8±16.6% to 102.2±13.2%; free protein S: 39.6±5.7% to 39.2±5.4%). In contrast, in a 64-year-old non-renal patient with iron deficiency anaemia who was treated with oral ferrous sulphate, protein C levels fell from 79% to 67% and total protein S levels fell from 101% to 72% (free protein S: 33% to 21%) over a 10-week period during which his haemoglobin rose from 4.9 to 9.9 g/dl.

Fistula blood flow

There was no significant change in fistula blood flow over the first 4 months of EPO treatment (Table 10). At 12 months, this showed a tendency to increase but results were not statistically significant.

DISCUSSION

The results presented here show a marked reduction in both protein C and protein S levels in two groups of haemodialysis patients treated with EPO. It is well-established that protein C and protein S are important endogenous inhibitors of coagulation in plasma, being synthesised in the liver by vitamin K-dependent mechanisms [213,214,215,216,217]. The inactive precursor of protein C is converted to the active protease by thrombin and an endothelial cell-associated cofactor called thrombomodulin [218]. Activated protein C has a potent anticoagulant effect by inactivating factors V and VIII [214] and stimulating the fibrinolytic system [219]. Protein S acts as a cofactor by enhancing the rate of inactivation of factors Va and VIIIa by protein C. It has been postulated that this is effected by promoting the binding of protein C to phospholipid [220]. Protein S exists in two forms in plasma: 60% is bound

to the complement component C4b-binding protein, and the remainder is free. Only the latter fraction appears to be active [221].

Both congenital and acquired protein C deficiencies are associated with a thrombotic tendency [222,223,224,225]. More recently, protein S deficiency has also been discovered to predispose to thrombotic events [221]. The levels of protein C and protein S obtained after 4 months of EPO therapy in this study are comparable to those levels known to promote thrombosis [212,213]. It is possible, therefore, that the higher-than-expected incidence of fistula thrombosis in patients receiving EPO may be in part related to the reduction in levels of these natural anticoagulants during the first few months of therapy. Consistent with this is the observation that clotting of the fistula usually occurs early in the course of EPO therapy. It is also interesting that the two patients in this study who thrombosed their fistulae did so when their protein C and protein S levels were at their lowest, and furthermore that these two patients had the greatest falls in these factors.

Whether the reduction in protein C and protein S is a direct effect of the EPO *per se* or whether it is a consequence of the correction of anaemia is still not clear. There was no change in either protein C or protein S levels after acute correction of renal anaemia by transfusion in this study. However, in the non-renal patient with iron deficiency anaemia who had a more gradual rise in haemoglobin concentration with iron therapy, there appeared to be a significant fall in both protein C and protein S. It is, therefore, possible that slow amelioration of anaemia results in a transient fall in levels of protein C and protein S which is not seen with more rapid correction. Further evidence against a direct effect of EPO is found in one patient who had a poor haemoglobin response to EPO and latterly had to be withdrawn from the study due to gastrointestinal bleeding. She showed no change in her protein C or protein S levels over 4 months despite receiving higher doses of EPO than the other patients in this study. Although results from this one patient have to be interpreted with caution, this again is consistent with a hypothesis that the EPO-induced reduction in protein C and protein S occurs

secondarily to the improvement in anaemia rather than to the EPO therapy itself. The exact mechanism by which this occurs, however, is uncertain at the present time.

Uraemic patients on long-term haemodialysis are prone to both a bleeding tendency and a hypercoagulable state [226,227,228,229]. This paradoxical association is not clearly understood, but several studies have sought to elucidate this by investigating the role of platelets and platelet function [226,227], coagulation factors [230], the fibrinolytic system [228], anti-thrombin III [231,232,233], factor VIII complex [229], and protein C [234,235]. The results of these studies, however, are confusing. Sorensen et al. [234] found depressed protein C activity in 14 haemodialysis patients compared to age- and sex-matched controls. In a later study, the same group confirmed their earlier results, but found normal protein C antigen levels in 10 haemodialysis patients [235], consistent with the results obtained from the patients in the present study prior to EPO therapy. They postulated that one or more inhibitors of protein C activity may be present in uraemic plasma.

The striking improvement in bleeding time reported here confirms the earlier findings of Moia et al. [206], and shows in addition that this improvement is maintained in the long-term. The exact mechanism for this effect is not yet fully elucidated, but it seems clear that the presence of adequate numbers of red cells are required for normal platelet function [236].

It was reassuring to find no reduction in fistula blood flow over the first 12 months of EPO therapy despite a significant increase in whole blood viscosity. Nevertheless, in none of the patients were any pre-existent stenotic areas identified which might be expected to lead to thrombotic problems in the presence of a greater blood viscosity. More disappointing was the apparent inability of the Doppler ultrasound technique to predict an impending fistula thrombosis in the two patients in which this occurred. Both patients had had an assessment of their fistula only 1-2 weeks prior to its occlusion, and there was no hint of any reduction in blood flow. This suggests that the occlusion happened fairly rapidly, rather than developing gradually in a slowly worsening stenosis.

Whether prophylactic anti-thrombotic measures are warranted in haemodialysis patients receiving EPO remains uncertain, although there are theoretical reasons for using an anti-platelet agent in those at high risk because of other factors [237]. Further studies will be required to elucidate the exact mechanism of the reduction in protein C and protein S levels in patients receiving EPO, and the role this has in predisposing to thrombosis.

Table 9 Effect of EPO on bleeding time in 10 haemodialysis patients treated with an initial dose of 240 U/kg/wk IV

PATIENT	Pre-EPO	+6 wks	+16 wks	+1 yr
SW	12	12	6	5
HL	30+	7	3	6
BB	30+	12	6	6
RP	30+	30+	11	8
EE	7	4	4	5
SR	30+	30+	6	11
AF	30+	14	6	6
CL	13	4	6	6
AG	30+	13	9	5
YJ	30+	30	6	6

Results expressed in mins (normal <10 mins).

30+ = greater than 30 mins.

(NB: SR on dipyridamole throughout).

Table 10 Haemostatic parameters and fistula blood flow measured in 10 haemodialysis patients during EPO therapy

		Time (weeks)				
		0	4	8	12	16
OSPT (secs)		16.5 (1.3)	16.2 (1.3)	16.5 (1.1)	14.9 (1.1)	15.5 (0.5)
KCCT (secs)		38.9 (2.4)	38.1 (3.2)	37.4 (2.9)	38.4 (3.2)	38.3 (3.9)
WBCT (mins)		7.5 (2.2)	6.9 (0.8)	6.6 (1.9)	6.7 (1.7)	6.9 (1.3)
PCI (%)		19.6 (11.1)	15.0 (11.5)	16.6 (7.8)	16.6 (11.8)	21.3 (15.0)
Fibrinogen (mg/dl)		276 (44)	326 (83)	296 (52)	304 (65)	298 (72)
Factor VII (%)		147 (31)	150 (43)	113 (18)	164 (31)	145 (19)
Factor VIII (%)		101 (42)	115 (27)	120 (38)	134 (31)	115 (27)
AT III (%)		102 (13)	103 (17)	107 (26)	105 (27)	87 (8)
Platelet aggregability to ADP (mm)	$5x10^{-7}$ M	0.1 (0.4)	0 (0)	0 (0)	0.8 (1.4)	0 (0)
	$5x10^{-6}$ M	21.1 (17.6)	17.3 (13.2)	0.3 (0.7)	5.9 (10.9)	20.4 (10.0)
	$5x10^{-5}$ M	32.4 (15.3)	30.0 (7.2)	28.5 (16.3)	16.8 (12.3)	25.8 (11.4)
Fistula blood flow (l/min)		1.02 (0.35)	1.03 (0.28)	1.06 (0.30)	1.08 (0.31)	1.09 (0.23) [1.42 (0.44) at 12 mths]

Results expressed as means (SD).

OSPT = one-stage prothrombin time

KCCT = kaolin cephalin clotting time

WBCT = whole blood clotting time

PCI = prothrombin consumption index

AT III = anti-thrombin III levels

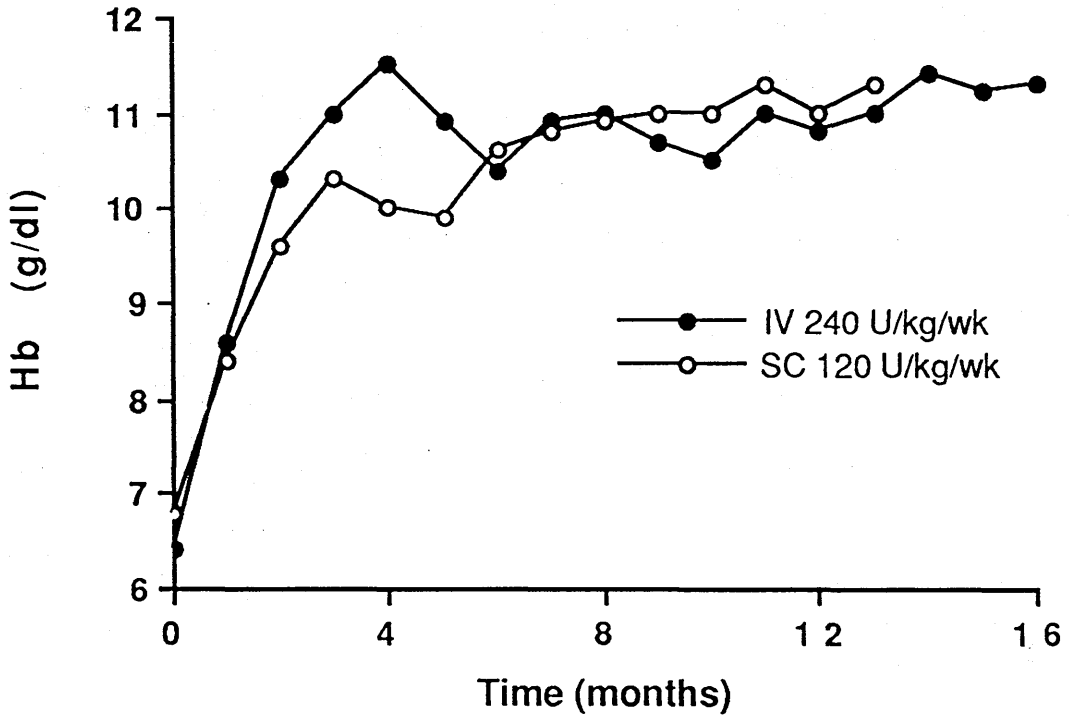


Fig. 34 Mean haemoglobin response to EPO therapy in 2 groups of haemodialysis patients:-

Group 1 treated with 240 U/kg/wk of EPO given intravenously.

Group 2 treated with 120 U/kg/wk of EPO given subcutaneously.

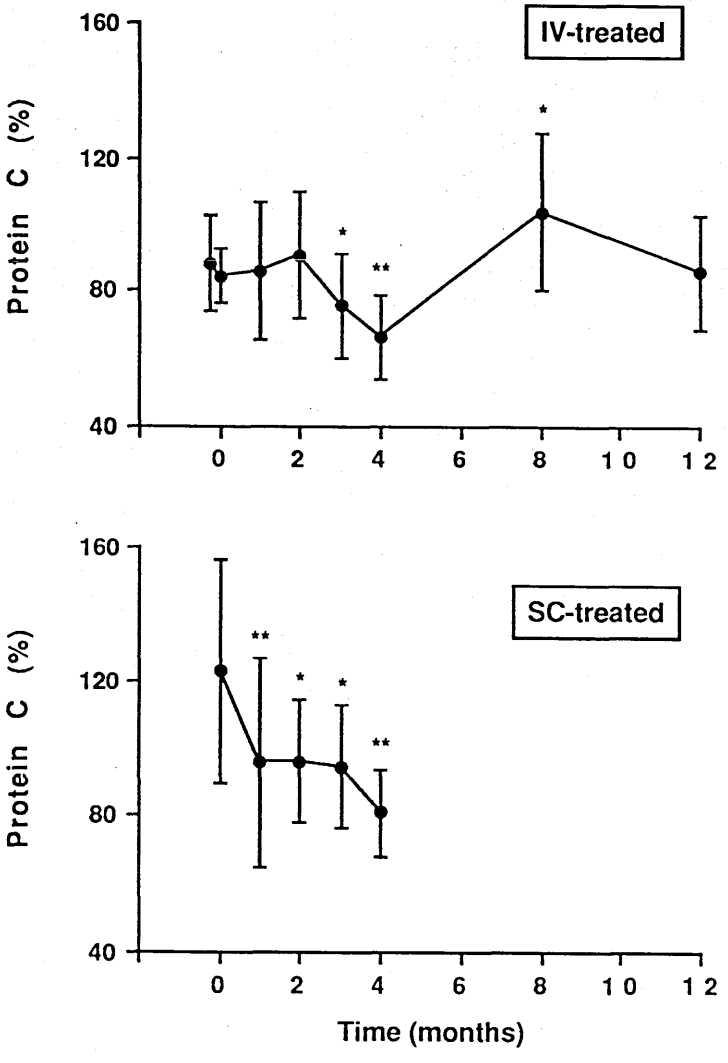


Fig. 35 Effect of EPO treatment on protein C levels in plasma.

Top figure: results for Group 1 patients treated intravenously.

Bottom figure: results for Group 2 patients treated subcutaneously.

Results expressed as means ± SD.

*p<0.05, **p<0.005 compared to baseline (paired t tests).

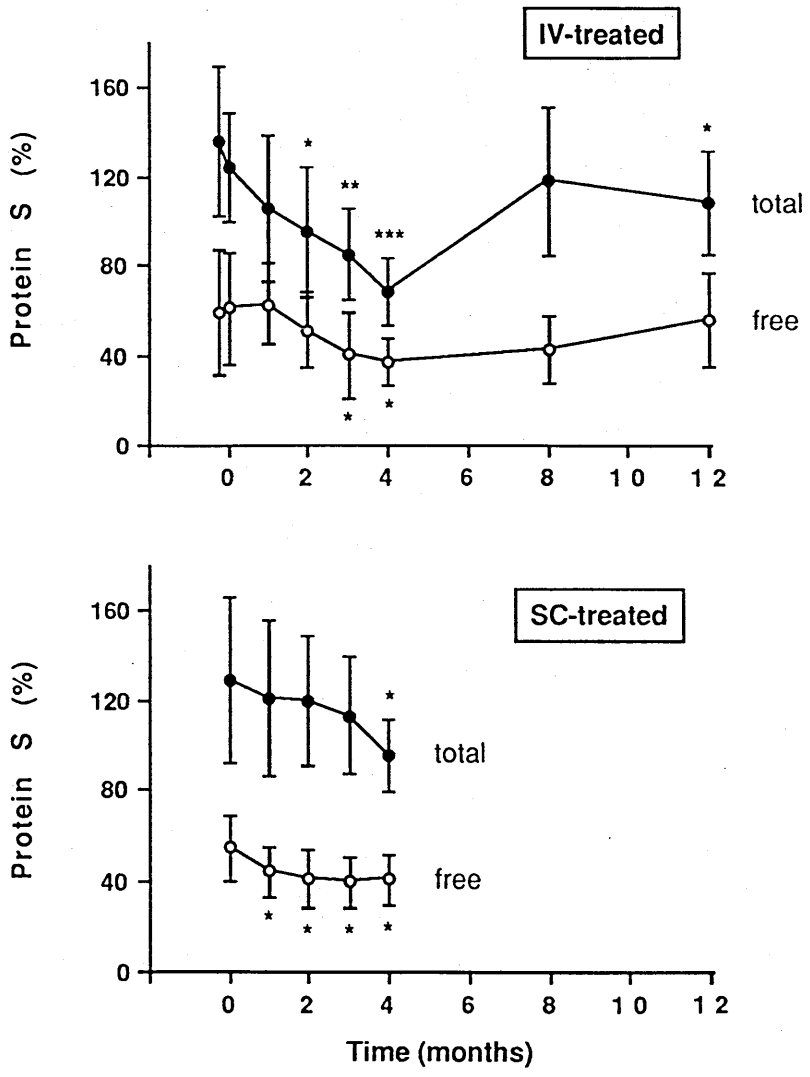


Fig. 36 Effect of EPO treatment on total and free protein S levels in plasma.

Top figure: results for Group 1 patients treated intravenously.

Bottom figure: results for Group 2 patients treated subcutaneously.

●——● total protein S

○——○ free protein S

Results expressed as means ± SD.

*p<0.05, **p<0.01, ***p<0.0005 compared to baseline (paired t tests).

CHAPTER 6

STUDIES USING ^{125}I-LABELLED RECOMBINANT HUMAN ERYTHROPOIETIN

INTRODUCTION

The studies described in the previous chapters of this thesis sought to elucidate the biological effects of amelioration of renal anaemia with recombinant EPO in dialysis patients. In the present chapter, a number of studies are described which make use of ^{125}iodine-labelled EPO as a tool for examining the metabolic fate of erythropoietin, along with the absorption kinetics of subcutaneously-administered EPO.

Details of the labelling procedure are given in full, and some of the problems encountered with interpreting the subsequent behaviour of the radiolabelled product are also described. Before conducting detailed studies with ^{125}I-EPO, it was clearly necessary to show that it had the same structural and biological characteristics as unlabelled EPO. Thus, chromatographic and electrophoretic techniques, along with a standard bioassay, were used to analyse the labelled material.

There are several theoretical advantages in using a radiolabelled preparation of EPO. Firstly, it is relatively quick and easy to calculate pharmacokinetic parameters following an IV bolus injection by measuring serial counts of activity in the plasma or serum. Assessing urinary counts gives information on renal handling and clearance, and by performing chromatographic analysis of the serum samples it is possible to look for metabolites of EPO which might retain radioactivity but be non-immunoreactive. Furthermore, in the second study, using radiolabelled EPO allowed the disappearance of the drug from subcutaneous injection sites to be monitored by means of an external gamma counter. This could be correlated with the appearance of counts in the bloodstream, and comparisons made between different injection sites.

The chapter is divided into 3 main sections:-

(i) Labelling of EPO with ^{125}iodine.

(ii) Kinetics and clearance of intravenously-administered ^{125}I-labelled EPO in healthy volunteers.

(iii) Absorption kinetics and bioavailability of subcutaneously-administered ^{125}I-labelled EPO: comparison of three different sites of injection.

(i) Labelling of EPO with ^{125}iodine

The chloramine-T method has been used widely in the past for labelling proteins with radioactive iodine [238]. In order to be able to perform the studies described later, this method was therefore used for labelling EPO with ^{125}iodine. Various chromatographic and electrophoretic tests were carried out to characterise the nature of the radiolabelled material, and its bioactivity was confirmed by the polycythaemic mouse bioassay.

Methods & Results

Chloramine-T method: 50 µl of chloramine-T (0.5 mg/ml) (Sigma Chemical Co. Ltd., Poole, Dorset, UK) in 0.05 M phosphate-buffered saline (PBS) was added to 25 µg of carrier-free recombinant EPO, kindly supplied by Boehringer Mannheim GmbH, Germany. To this was added 10 µl of Na ^{125}I (100 mCi/ml; Amersham, Buckinghamshire, UK), i.e. 1 mCi or 37 MBq of radioactivity. The solution was mixed well for 30 seconds, and the reaction was then stopped by 100 µl of sodium metabisulphite (2 mg/ml) in PBS. At this point, 100 µl of 10 mg/ml potassium iodide was added as a carrier, followed by 200 µl of 2% human serum albumin.

Separation of ^{125}I-labelled EPO from unreacted ^{125}iodide was carried out using a 10 cm Biogel P60 desalting column (Bio-Rad, Thetford, Essex, UK). The column was primed using 2-3 ml of 2% human serum albumin (CIS UK Ltd., High Wycombe, UK) to reduce adsorption, and 0.5-1.0 ml of ^{125}I-labelled material was then loaded onto it and eluted with PBS. 1 ml fractions of elute were collected into 4 ml polystyrene tubes every 1-2 minutes, and the column run until both the ^{125}I-labelled EPO and free ^{125}iodide peaks had been eluted. The radioactivity in all fractions was measured, and the fraction containing the highest activity from the earlier peak was retained for further use; this generally eluted at about 4-5 ml.

The percentage yield was calculated from the activity in the tube divided by the initial activity introduced into the system (37 MBq), and multiplied by 100%. This

was generally found to be between 60 and 70%, and the specific activity of the radiolabelled material was usually around 1 MBq/μg of protein. Thus the end-result of the procedure was a fairly "light" labelling of approximately 1 atom per molecule of EPO in order to minimise the potential damage to the protein structure.

The ^{125}I-labelled material was then subjected to:-

a) **Gel permeation chromatography** using a G75 Sephadex column (Pharmacia, Milton Keynes, UK) measuring 1 metre in length. Both phosphate-buffered saline and sodium dodecyl sulphate (SDS) were used to elute the sample. The void volume (V_o) for the column was 32 ml and the V_t was 88 ml. 2 ml fractions of the elute were collected at a rate of 5 fractions per hour for 24 hours. The fractions were then counted on a gamma counter and the total counts per minute (cpm) in each fraction determined. The elution profile of level of radioactivity versus elution volume was then constructed. Fig. 37 shows a representative elution profile for the ^{125}I-labelled material run in PBS. Three peaks were obtained and previous calibration curves for this column suggested that the second peak (which constituted approximately 95% of the total activity) correlated with the known molecular weight of native EPO (30,400 daltons). The third peak represented residual free ^{125}iodine, but the nature of the higher molecular weight first peak (which constituted <5% of the total activity) was uncertain. The possibilities were that it represented a dimer of EPO (since its molecular weight corresponded to approximately 60,000 daltons), or EPO bound to some other substance. This phenomenon has been described in previous studies [72,239], and Sherwood et al. [239] have suggested that the first peak represents dimeric ^{125}I-labelled EPO. This peak was not present when the radiolabelled material was run in the same column using SDS (Fig. 38). This suggests that the binding of EPO either to itself or another substance is fairly loose and is easily abolished by SDS, again consistent with ionic- or hydrophobic-bound dimeric EPO. Spivak & Hogans [82] similarly found that

when EPO was labelled with ^{125}iodine using the lactoperoxidase technique, a single band of radiolabelled protein was obtained when analysed by either electrophoresis in SDS-urea/7.5% polyacrylamide tube gels or 12.5% SDS-polyacrylamide slab gels.

Another problem encountered during the column work was the recovery of radiolabelled material in the eluate. This was as low as 31-58% (mean 42.6%) when the ^{125}I-EPO was dissolved in PBS alone, but if dissolved in human serum to provide additional carrier protein, the recovery increased to 89-99% (mean 94.7%). This is almost certainly due to the fact that much of the ^{125}I-EPO was adsorbed on the glass tube unless sufficient carrier protein was present. The likely explanation for this is the extreme hydrophobicity of EPO as described earlier (see Literature Survey, section 4: Biochemistry and physiology of erythropoietin).

b) **SDS-polyacrylamide gel electrophoresis.** The electrophoretic mobility of the radiolabelled material in a 12% gel is shown in Fig. 39 and is compared with that of a number of protein standards of known molecular weights. More than 98% of the ^{125}I-labelled material was concentrated in a single band occurring between carbonic anhydrase (MW 30,000 daltons) and ovalbumin (MW 43,000 daltons), and this again is consistent with the known molecular size of native EPO.

(i i) Kinetics and clearance of intravenously-administered ^{125}I-labelled EPO in healthy volunteers

Previous pharmacokinetic studies as described in Chapter 1 and elsewhere [77,105,108,240] suggest that EPO clearance conforms to a one- or two-compartment model. There is, however, virtually no information on the metabolism of the

intravenously-administered recombinant hormone [71]. This was therefore studied in a group of normal healthy subjects.

The EPO was labelled with ^{125}I as described above. Six normal healthy volunteers (3 males, 3 females; age range 23-44 years) drawn from the hospital staff were studied. Each was given an injection of 1 MBq as a single intravenous bolus. All subjects took oral potassium iodide, 60 mg daily for 7 days, commencing 24 hours prior to injection in order to block thyroid uptake of ^{125}iodine. Blood samples were taken at 0, 15, 30 and 45 minutes, and 1, 2, 3, 4, 6, 8, 12, 18, 24, 36, 48, 60, 72, and 96 hours. Eight 12-hour urine collections were obtained over this 4-day period in order to determine urinary clearance of EPO. Both serum and urine samples were assayed for protein-bound and free iodide counts.

The serum protein-bound activity curve was plotted on a semi-log scale against time (Fig. 40). Analysis of this curve suggested three exponential phases (alpha, beta, and gamma) with mean (±SD) half-lives of 1.48±0.19, 3.52±0.27, and 26.3±3.1 hours, respectively. More than 95% of circulating counts were protein-bound. Urine analysis showed that there was a negligible excretion of protein-bound counts. The apparent volume of distribution was 9.48±1.18 litres.

The half-life data in this study were quite different from previous pharmacokinetic studies of EPO [77,105,108,240]. This is likely to be due to the presence of dimeric EPO produced by the labelling procedure. Further evidence for this is shown in Fig. 41 in which gel permeation chromatography of serum from one of the subjects was carried out for the samples at 0.5, 6, 12, 24, and 48 hours. This shows a much more rapid decay of the peak 2 activity which would be consistent with monomeric EPO, in contrast to peak 1 activity (dimeric EPO) which declined more slowly and thus assumed a much greater proportionate representation of the total serum activity. The gamma phase in this study therefore seems likely to be due to the persistence of the slowly-cleared higher molecular weight peak 1 activity in serum, which was probably an EPO dimer.

Since there has been no gamma phase reported in any of the pharmacokinetic studies in which EPO has been measured by radioimmunoassay, it would appear that this putative dimer is non-immunoreactive. The lack of any additional peaks in the elution profile suggests that there is no further aggregation of EPO in the bloodstream, nor is there any evidence of smaller molecular weight metabolites of EPO. What is not clear from this study is whether EPO exists as a dimer under physiological conditions or whether this has occurred as a consequence of the labelling procedure. It is also uncertain whether there is a dynamic equilibrium between dimeric and monomeric labelled EPO in the circulation. Since there is no evidence in this study of EPO metabolites in circulation, it is possible that the plasma elimination of EPO occurs as a consequence of tissue or organ uptake of the hormone rather than by true catabolism of EPO.

(iii) Absorption kinetics and bioavailability of subcutaneously-administered ^{125}I-labelled EPO: comparison of three different sites of injection

The data presented in Chapter 2 suggest that the subcutaneous route for administering EPO may confer several advantages over the IV route. Other published work also indicates that the SC route is rapidly gaining popularity as the preferred route of administration in both haemodialysis and CAPD patients [114,115,127,128]. There is, however, no information as to which is the optimum site for injecting SC EPO. It has previously been shown that absorption of subcutaneous insulin was fastest from the abdomen, intermediate from the arm, and slowest from the thigh [241,242]. In view of the cost of EPO, even small differences in bioavailability between different injection sites may result in considerable saving.

The aim of the present study was to compare the absorption kinetics and bioavailability of ^{125}I-labelled EPO injected subcutaneously into the upper arm, the abdomen, and the thigh in normal healthy volunteers.

Subjects and Methods

Eight subjects (4 males, 4 females; mean age 30±5(SD) years; mean weight 68±14 kg) were studied. Bulkware EPO (Boehringer Mannheim GmbH), which was free from stabilising and solubilising agents, was labelled with ^{125}I using the chloramine-T method as described earlier. All subjects took oral potassium iodide, 60 mg daily for 7 days, commencing 24 hours prior to injection in order to block thyroid uptake of ^{125}I. 0.5 MBq of ^{125}I-EPO was then injected SC into the upper arm (mid-deltoid region), the abdomen, and the thigh by the same operator on 3 occasions, separated by at least 10 days. Absorption of ^{125}I-EPO was monitored in two ways: (i) by its disappearance from the site of injection using an external gamma counter, and (ii) by its appearance in the bloodstream. Blood samples were taken at 0, 1, 2, 4, 8, 12, 18, 24, 36, 48, 72, and 96 hours after injection, and protein-bound counts determined using a resin which adsorbs free iodide. The thickness of the subcutaneous tissue at each injection site was measured by ultrasound on a Toshiba Sonolayer SSA-100A system (Crawley, West Sussex, UK) in order to correlate bioavailability with the degree of SC fat.

Results

The radioactivity monitored externally over the injection site diminished more rapidly following injection into the thigh than for the other two sites (Fig. 42). The disappearance profiles for the arm and abdomen were comparable. After 4 days, significantly less ^{125}I-EPO remained in the subcutaneous tissue after thigh injection compared to the other two sites (Fig. 42, Table 11).

Serum counts (cpm per 1 ml of serum) rose faster after injection into the thigh than for the arm or abdomen (Fig. 43). Mean peak levels were nearly twice as high for the thigh as for the other two sites (Table 11). The time taken to reach peak blood levels was somewhat variable among subjects, and overall there was no significant difference between the three injection sites. Likewise, the subsequent elimination from the plasma compartment was fairly comparable for all three sites. The area under the curve (AUC) to 96 hours for the serum profile following thigh injection, however, was significantly

greater than for the arm ($p<0.01$) and abdomen ($p<0.005$). When the AUC was extrapolated to infinity, however, the difference between the thigh and the arm became less marked, both sites being superior to the abdomen (Table 11). Although there was some variability in the thickness of subcutaneous tissue both between subjects, and between different sites in the same subject (Fig. 44), there was no significant correlation between the SC tissue thickness and the bioavailability (Fig. 45).

Discussion

Despite increasing usage of the subcutaneous route for administering EPO [114,115,116,127,128], no studies have been published which compare different sites of SC injection. The results of the present study, which although conducted in normal healthy volunteers rather than patients, suggest that the thigh may be the optimal site of injection. Absorption from this site was more rapid, and higher peak serum levels and bioavailability were obtained. This is in striking contrast to what has been previously reported for subcutaneous insulin where absorption was faster for the abdomen than for the arm, which was, in turn, faster than the thigh [241,242]. The possible reasons for this discrepancy are not clear at the present time, although EPO is very different in molecular size and composition to insulin, being larger and more heavily glycosylated. Also, the mobility of the subjects in our study was probably greater than for those used in the insulin studies. It is likely that exercising the lower limb, by increasing regional blood flow, would improve absorption from subcutaneous tissue. No restriction was put on our subjects as far as exercise was concerned; they were instructed to carry out their daily activities as usual. It is possible, however, that EPO-treated patients would be less fit, and have correspondingly lower rates of absorption from the thigh, but this requires validation.

In conclusion, on the basis of these preliminary results, the optimal site for SC administration of EPO may be the thigh. Further studies, however, are required to confirm this, particularly in patients receiving haemodialysis or CAPD.

Table 11 *Summary* of pharmacokinetic data obtained following injection of ^{125}I-labelled EPO into 3 different subcutaneous sites

Site	Peak serum level (cpm)	Time to peak (hr)	k_{el}	AUC to 96 hr	AUC to infinity	% left at injection site at 96 hr
THIGH	809 ±354	13.5 ±6.7	0.015 ±0.007	42376 ±9751	60907 ±13332	8.0 ±4.3
ARM	525* ±120	13.5 ±8.8	0.010 ±0.003	33301** ±6576	54301 ±8853	14.1* ±5.0
ABDOMEN	502* ±98	18.8 ±5.9	0.013 ±0.002	31788*** ±7511	46621*** ±9451	12.2 ±4.7

*p<0.05, **p<0.01, ***p<0.005 compared with results for thigh

Results expressed as means ± SD

cpm = counts per minute; k_{el} = elimination constant; AUC = area under the curve

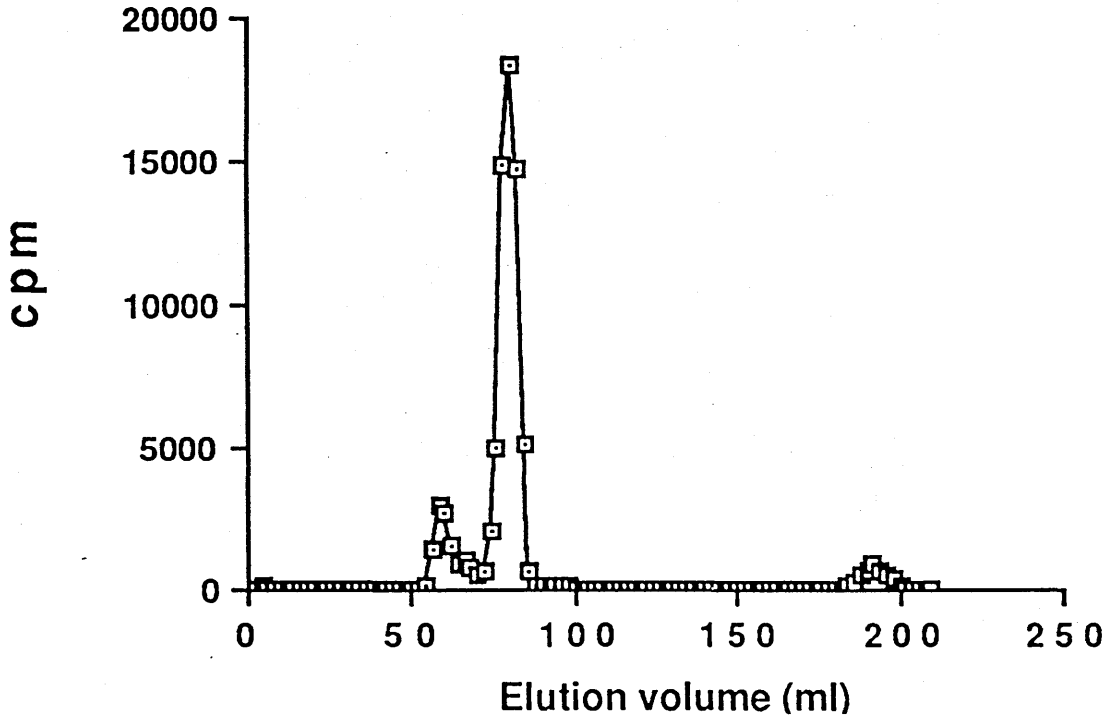


Fig. 37 Elution profile of ^{125}I-labelled EPO eluted in phosphate-buffered saline from a G75 Sephadex column.

cpm = counts per minute.

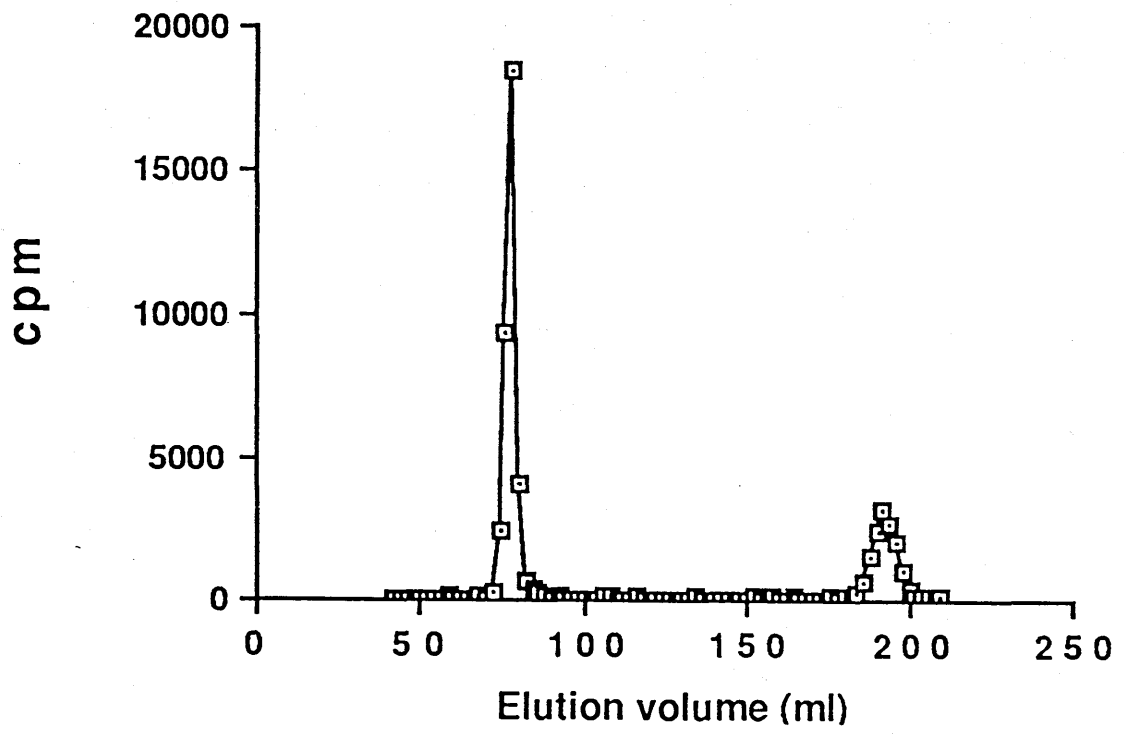


Fig. 38 Elution profile of ^{125}I-labelled EPO eluted in sodium dodecyl sulphate from a G75 Sephadex column.

cpm = counts per minute.

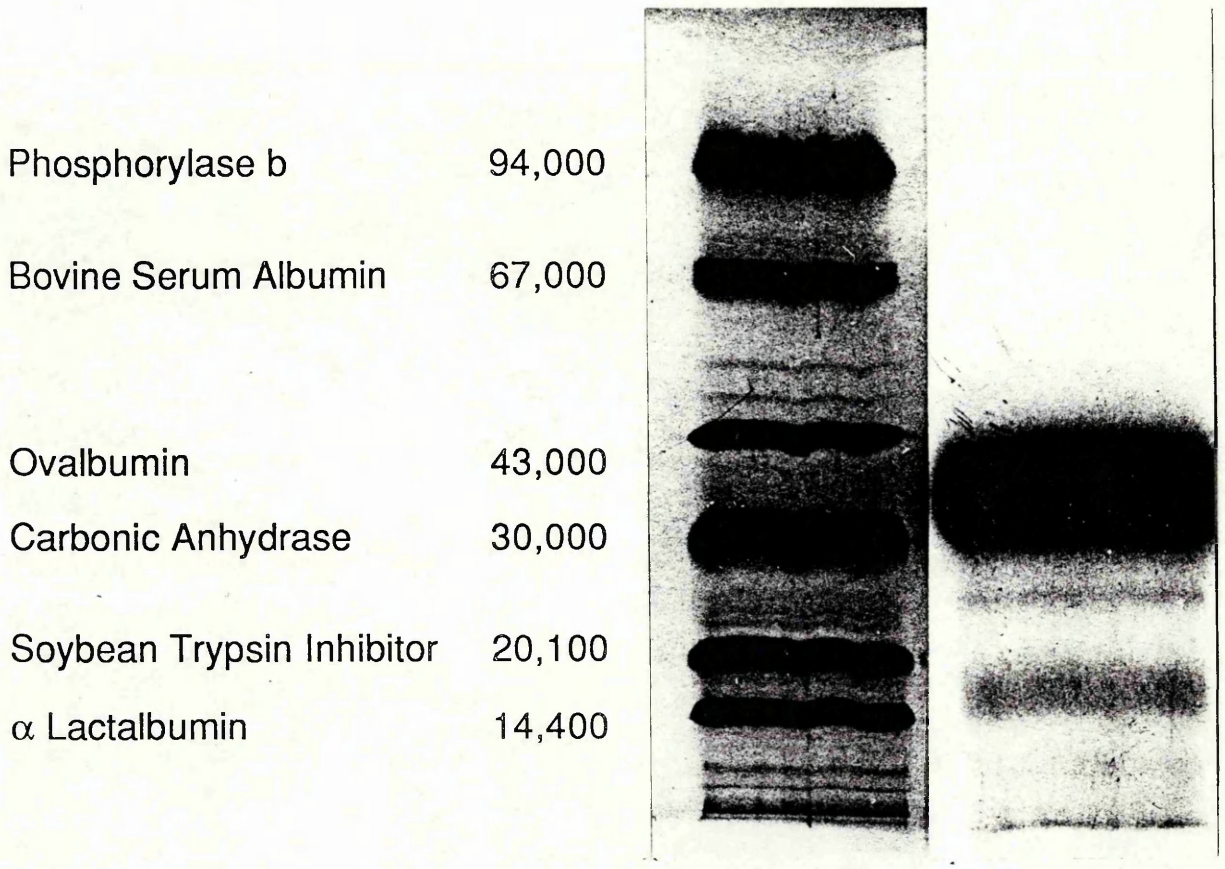


Fig. 39 Polyacrylamide gel electrophoresis of ^{125}I-labelled EPO in sodium dodecyl sulphate.

Left-hand strip shows calibration bands of a number of protein standards of known molecular weights (in daltons) as shown.

Right-hand strip shows a dense band of ^{125}I-labelled EPO lying between carbonic anhydrase and ovalbumin.

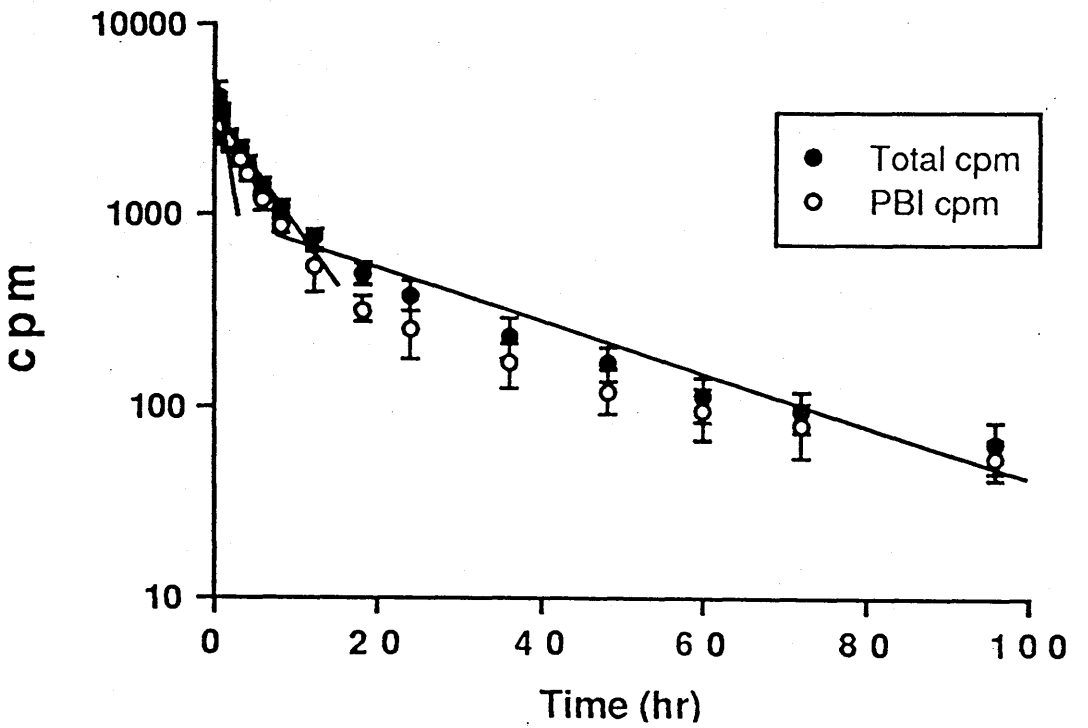


Fig. 40 Serum ^{125}I-labelled EPO profile following IV injection of 1 MBq to 6 normal healthy volunteers.

Results are shown both as total counts per minute (cpm) and protein-bound (PBI) cpm in 1 ml samples of serum, and are expressed as means ± SD.

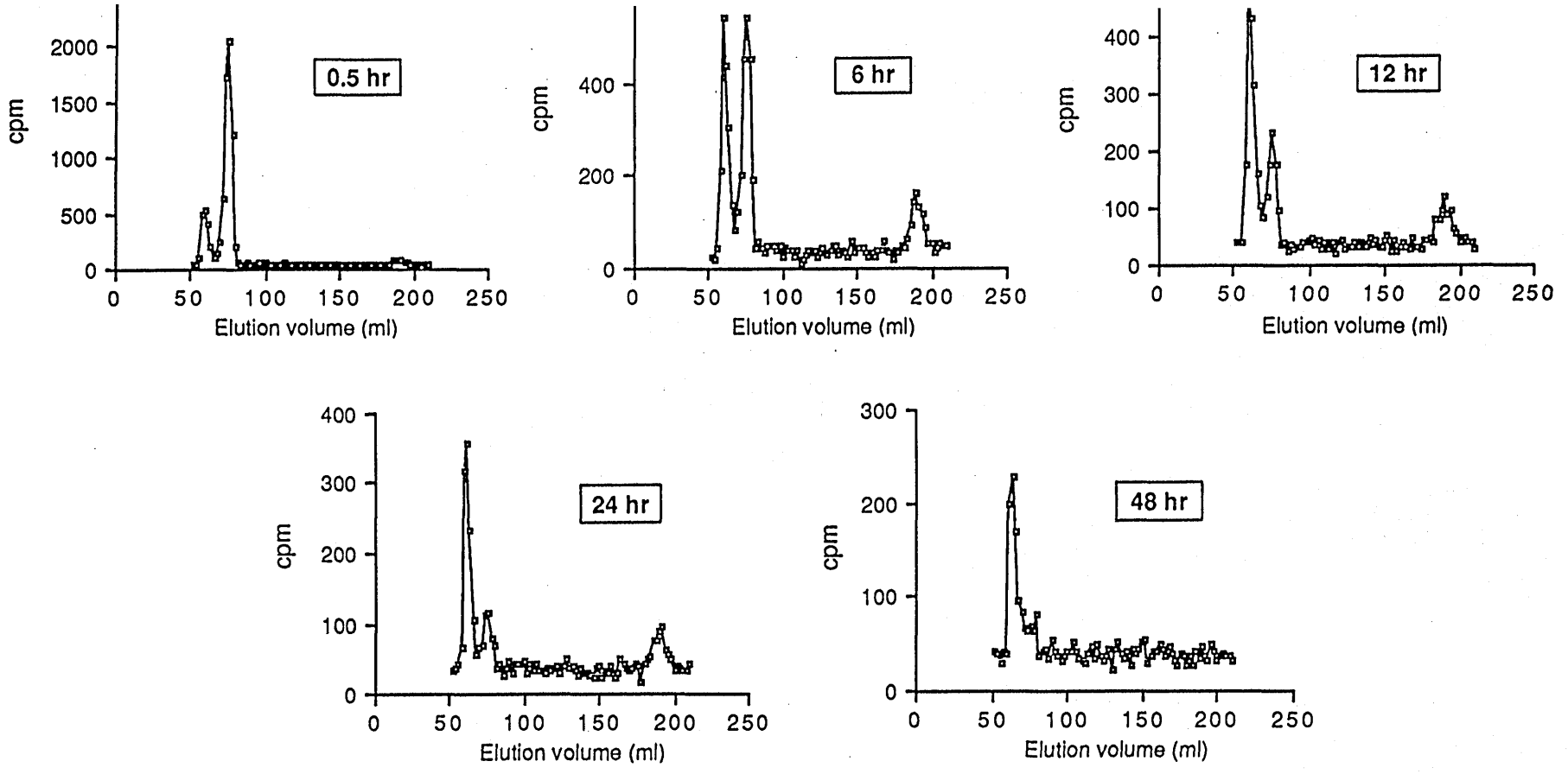


Fig. 41 Gel permeation chromatography of serum samples obtained 0.5, 6, 12, 24, and 48 hours following IV injection of 1 MBq of ^{125}I-labelled EPO to a single normal healthy subject.

cpm = counts per minute.

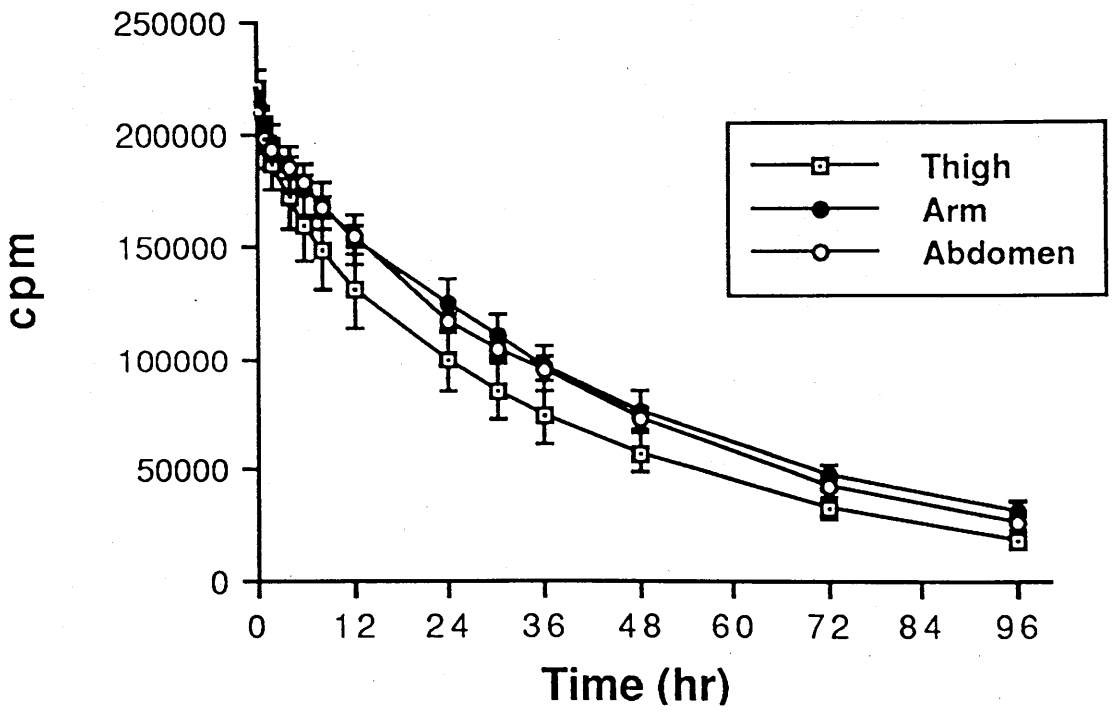


Fig. 42 Comparison of disappearance of radioactivity from 3 subcutaneous sites following injection of 0.5 MBq ^{125}I-labelled EPO.

Results expressed as means ± SE.

cpm = counts per minute.

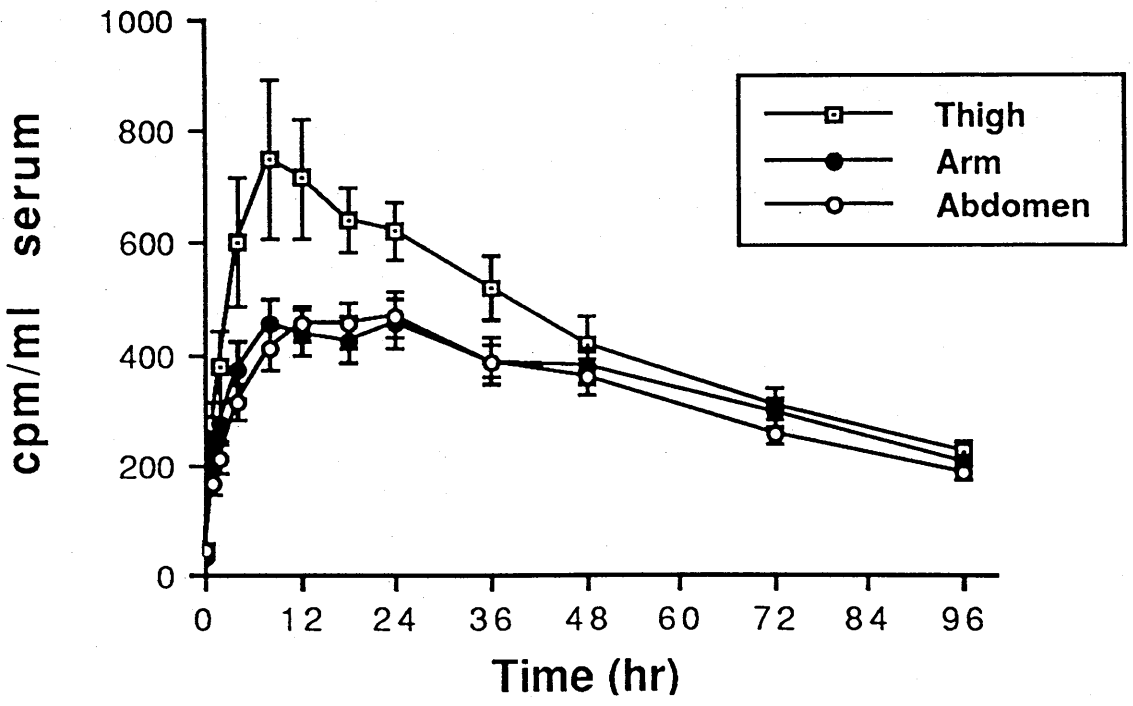


Fig. 43 Comparison of serum ^{125}I-labelled EPO profiles following injection of 0.5 MBq into 3 different subcutaneous sites.

Results expressed as means ± SE.

cpm = counts per minute.

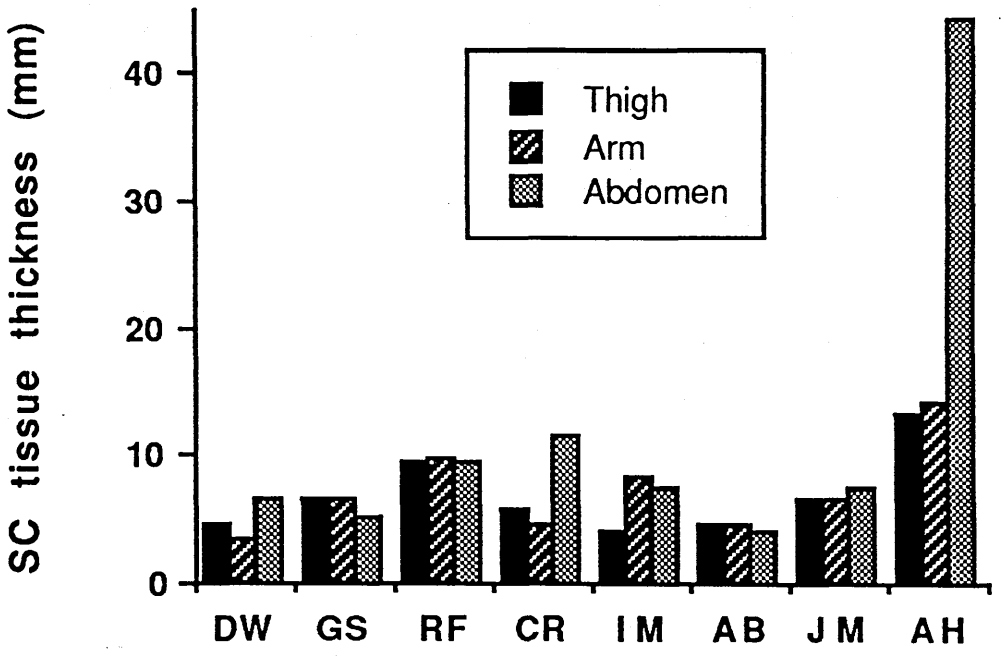


Fig. 44 Thickness of the subcutaneous (SC) tissue measured by ultrasound at the 3 injection sites in 8 normal healthy volunteers.

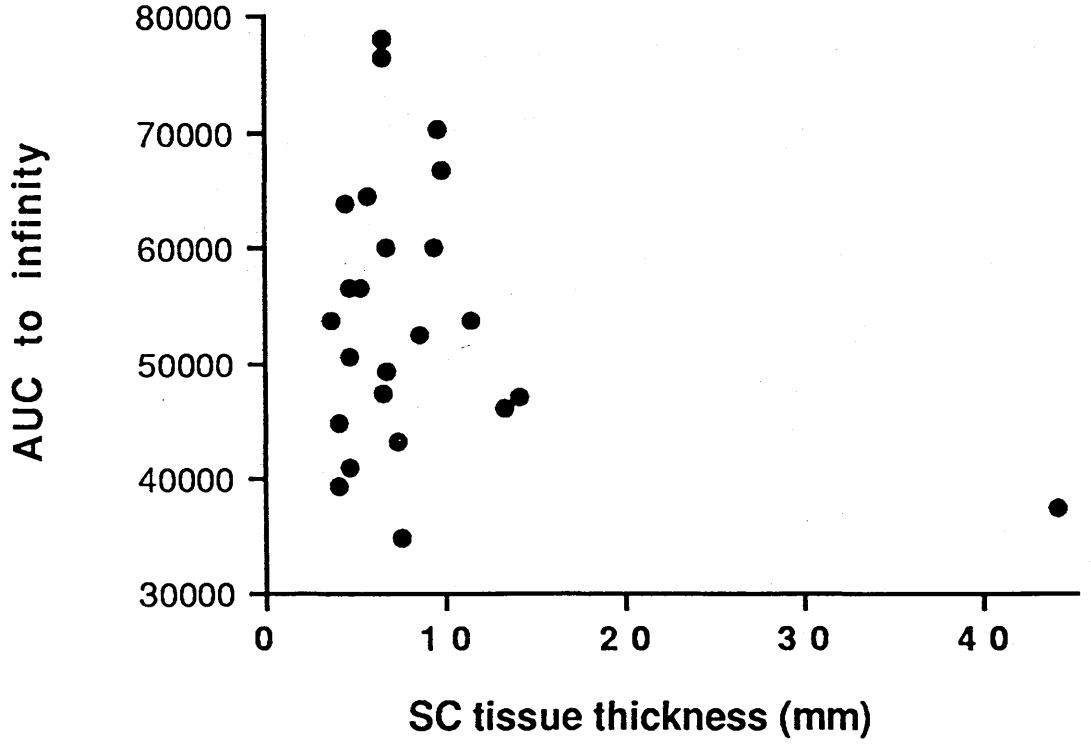


Fig. 45 Correlation between a measure of bioavailability (the area under the serum concentration-time curve (AUC) extrapolated to infinity) and the thickness of the subcutaneous (SC) tissue following injection of ^{125}I-labelled EPO into 3 different SC sites in 8 normal healthy volunteers.

CHAPTER 7

TREATING RENAL ANAEMIA WITH RECOMBINANT HUMAN ERYTHROPOIETIN : PRACTICAL GUIDELINES AND A CLINICAL ALGORITHM

INTRODUCTION

Recombinant human erythropoietin is of proven benefit in the treatment of renal anaemia [1,2,3,4] and numerous clinical trials have indicated an acceptable degree of safety [90,91,92,93,159]. It has recently been licensed for use in the United States of America and several European countries including the United Kingdom. Thus the drug is now widely available, prompting the need for practical guidelines to ensure that patients receive maximal benefit with the least risk and greatest cost-efficiency.

Based on the author's own experience in treating 42 patients as well as on published studies, the following recommendations have been compiled together with a clinical algorithm as a guide for the management of patients receiving erythropoietin therapy. This chapter considers which patients should be treated, the optimal starting dose and route of administration, the monitoring of iron status, target haemoglobin concentration, the rate of response, and the investigation of an impaired response, as well as potential complications.

Which patients should receive erythropoietin therapy?

It remains uncertain exactly how many dialysis patients would benefit from EPO therapy, but estimates of between 50 and 75% have been made [2,3]. Since the treatment is both expensive and long-term, careful consideration has to be given to the selection of appropriate patients.

As there is often a rise in haematocrit after starting dialysis [28], particularly with CAPD [20,41], it is wise to wait at least three months after commencing renal replacement therapy before considering erythropoietin. Furthermore, if the patient is likely to receive a live donor transplant in the near future, EPO treatment might not be warranted. The avoidance of blood transfusion with EPO, however, may justify its use in this context by preventing cytotoxic antibody formation and hence improving the chances of a successful graft outcome. Patients with a haemoglobin concentration of greater than 10 g/dl are unlikely to have a noticeable improvement in well-being and have a higher chance of side-effects, since whole blood viscosity rises rapidly with higher levels of

haematocrit (see Chapter 4) [173,185,187]. Thus only uraemic patients with haemoglobin concentrations less than 10 g/dl should be considered for treatment.

It is important to exclude other causes of anaemia such as haematinic deficiency, blood loss, haemolysis, infection, aluminium toxicity, and malignancy. Any such disorder should be corrected and the haemoglobin response assessed before considering EPO therapy. In the absence of any other cause, patients with a haemoglobin concentration below 8 g/dl will almost certainly benefit from treatment. Those with a haemoglobin concentration of 8 to 10 g/dl who have symptoms referable to their anaemia, particularly angina, should also be offered EPO therapy.

Whether pre-dialysis patients should routinely be treated is still unclear. There is a theoretical risk that raising the haematocrit would, by increasing viscosity and reducing renal plasma flow, impair renal perfusion and thus lead to a more rapid decline in renal function [160]. However, studies to date suggest that erythropoietin therapy has no effect on the natural history of the uraemia [116,240,243]. In our experience, patients with a haemoglobin concentration of less than 8 g/dl usually have either end-stage renal failure or another condition contributing to their anaemia such as myeloma. Treatment should, therefore, normally be reserved for individuals with symptomatic anaemia who are unlikely to need to start long-term dialysis within the next three months.

Whether EPO therapy will be effective in patients with a multifactorial cause for their anaemia, for example uraemia and rheumatoid arthritis, is uncertain. The finding of an inappropriately low serum erythropoietin concentration for the degree of anaemia [9] could be an indication for a trial of treatment, but the response may be impaired by the underlying chronic disease. Further experience with the use of EPO in multifactorial anaemia is required before this question can be fully answered since most of the EPO trials to date excluded such cases.

Monitoring iron status before and during treatment with EPO

It is important to determine clearly the baseline iron status of any patient being considered for erythropoietin therapy for two reasons.

Firstly, if a patient is frankly iron deficient then one can expect some improvement in the haemoglobin concentration with iron therapy alone, either oral [244] or intravenous [245]. The potential cost saving following such simple therapy could be substantial. As a result, EPO treatment should be withheld until it is clear that the patient is not iron deficient, or until a deficiency has been fully treated as judged by a stable haemoglobin concentration. If the serum ferritin level is less than 15 µg/l, the patient is iron deficient. Higher concentrations of serum ferritin may be found in iron deficient patients, for example in conditions causing hepatocyte dysfunction or greatly increased iron resorption from extravasated blood. In these circumstances, the serum ferritin may be raised out of proportion to the iron stores. In addition, the transferrin saturation may also be acutely raised. If there is doubt about the patient's true iron status a therapeutic trial of oral or intravenous iron should be given for a minimum of four weeks. If there is any response during this period then EPO therapy should be postponed until a new stable haemoglobin concentration is achieved. At this point the need for EPO should be reassessed. In all cases it is important to remember that infection or active inflammatory processes, including peritonitis in CAPD patients, may prevent a response to iron therapy.

The second reason why it is important to estimate the baseline iron status of a patient starting EPO therapy is to determine whether there is enough readily available iron to meet the anticipated demand. The advent of erythropoietin has resulted in an unprecedented and potent therapeutic stimulus to erythropoiesis, and it has become apparent that large quantities of iron are utilised during this process. Thus, patients who are iron replete before starting EPO can rapidly become deficient under the influence of this treatment [1,2,91,123,124,125]. This may occur in the presence of a normal serum ferritin level (suggesting adequate iron stores) and stainable iron in the marrow, and the problem appears to be a limitation in the rate of iron supply, i.e. the stores are unable to release iron fast enough to meet the demand.

Previous work has shown that a rise of 1 g/dl in the circulating haemoglobin concentration uses 150 mg of storage iron (equivalent to nearly 20 µg/l of serum ferritin) [246]. Thus, for an anticipated haemoglobin rise of 5 g/dl following EPO

therapy, the absolute minimum requirements would be 750 mg of storage iron (100 µg/l serum ferritin). Patients with starting serum ferritin levels less than 100 µg/l, therefore, are highly likely to develop functional iron deficiency. Such individuals will require intensive iron supplementation, almost certainly in the form of parenteral iron. Recently, a nomogram was devised for estimating the projected iron deficit based on the initial haemoglobin and serum ferritin concentrations, and assuming a target haemoglobin level of 11.6 g/dl [123]. The same authors also demonstrated that oral iron supplementation was unlikely to be able to keep pace with the demand during the early phase of treatment with EPO [123].

Patients with initial serum ferritin levels greater than 100 µg/l may also develop functional iron deficiency [91,123] which may be detected from changes in the percentage transferrin saturation with iron. If this falls below 20% then it is likely that the available iron supply to the erythron is inadequate [1,2,123,124,125]. Bainton & Finch [247] previously demonstrated that functional iron deficiency occurred once the transferrin saturation was reduced to levels below 16%. Thus, if the transferrin saturation falls below 20% at any stage of EPO treatment, parenteral iron therapy is indicated. More recently, a new and more direct method of detecting functional iron deficiency has been reported [248]; the initial results are encouraging and further investigation is merited.

Figure 46 presents an algorithm for managing patients on EPO therapy, with particular emphasis on the monitoring of iron status and the need for iron supplementation.

What starting dose of EPO and which route of administration?

Most experience with EPO so far is with intravenous therapy in haemodialysis patients, and one of the earliest studies showed that there was a dose-dependent rate of response to erythropoietin [91]. However, it has become increasingly accepted that the risk of side-effects such as severe hypertension and thrombotic complications may be lessened with a haemoglobin rise not exceeding 1 g/dl/month. Most workers using intravenous EPO now prescribe an initial dose in the range of 100-200 U/kg/week for

haemodialysis patients, divided into 2 or 3 doses. A similar intravenous dosage regimen has been used with good effect in patients not yet on dialysis [116,240,243].

The intravenous route is clearly impractical for chronic use in CAPD patients who have no ready vascular access. Obvious alternatives to be considered included the intraperitoneal and subcutaneous routes. In a single-dose pharmacokinetic study in CAPD patients, the bioavailability of subcutaneously-administered erythropoietin was found to be seven times greater than that of intraperitoneal administration, but was still only 22% (see Chapter 1) [99]. These results were subsequently confirmed by Boelaert et al. [104]. Nevertheless, Frenken et al. [103] used the intraperitoneal route for treating 5 CAPD patients, and obtained an effective clinical response with a dose of 300 U/kg/week (3 separate administrations). Similar haemoglobin responses, however, were reported in 9 CAPD patients and 12 haemodialysis patients treated with only 120 U/kg/week (2 separate administrations) given subcutaneously [127,129]. This dose achieved a similar correction of anaemia to that obtained with 240 U/kg/week (2 administrations) given intravenously to a group of 10 haemodialysis patients [129]. The overall rate of response, however, was slightly slower.

Bommer et al. [114] showed that a 50% reduction in dose can be achieved when switching from intravenous to subcutaneous administration with no loss of effect. Stevens et al. [117] treated 12 CAPD patients with subcutaneous EPO and obtained a brisk response to 300 or 450 U/kg/week (3 administrations). The dose was then reduced to 37.5-150 U/kg (median 75 U/kg) weekly to maintain a haemoglobin concentration between 11.0 and 11.5 g/dl. A similarly rapid response was obtained in another study of five transfusion-dependent children on CCPD in whom the haematocrit increased from 22% to 33% after only 3 weeks' treatment with EPO given SC at an initial dose of 450 U/kg/week (3 administrations) [119]. A much lower SC dose of 100 U/kg/week (2 administrations) was used by Steinhauer et al. [118] to treat eight adult CAPD patients. In pre-dialysis patients, similar responses were achieved with 450 U/kg/week (3 administrations) intravenously and 300 U/kg/week (3 administrations) subcutaneously [116]. The lowest total weekly dose of EPO reported to be effective to date (98 U/kg/week) was also given via the subcutaneous route in a study by

Granolleras et al. [115]. These workers obtained an adequate haemopoietic response in haemodialysis patients with daily SC injections of only 14 U/kg.

Thus, the subcutaneous route appears to be gaining in popularity not only in CAPD patients [117,127] but also in haemodialysis subjects [115,129], and the evidence to date suggests that lower doses of EPO may be used when given by this route. Further dose-finding studies are required, but on the evidence available a starting subcutaneous dose in the range of 75-150 U/kg/week in 2 or 3 divided doses seems appropriate. If the patients can be taught to give their own injections, then the daily dosage regimen [115] may be worth considering.

What target haemoglobin concentration should be aimed for?

There is little doubt that it is possible to correct fully the anaemia of chronic renal failure with EPO. In balancing the benefits versus the risks, however, the common practice is to aim for partial correction. A linear increase in the haemoglobin concentration or haematocrit leads to an exponential rise in whole blood viscosity (see Chapter 4) [173,185,187] which, in turn, is thought to contribute to many of the side-effects of EPO therapy such as hypertension, increased peripheral resistance, and thrombotic complications. In addition, it appears that this partial correction of the anaemia causes near-maximal improvement in well-being, exercise capacity [154,158], and symptoms of anaemia. A further increase in the haemoglobin concentration does not seem to confer any additional symptomatic benefit [249], and would therefore be less cost-effective.

The optimal target haemoglobin concentration seems to be in the range of 10-12 g/dl. It is at this level that the risk:benefit ratio appears to be minimised though some flexibility is necessary in treating individual patients. Since the main aim of EPO therapy is to reverse the symptoms of anaemia, differing thresholds at which this occurs may influence the appropriate final haemoglobin concentration.

With regard to the rate of rise of the haemoglobin response, for most patients an increase of 1 g/dl/month appears to be a reasonable compromise. Exceeding this limit

may predispose the patient to an increased risk of side-effects, and there is rarely any indication for more rapid correction of anaemia.

What if there is an impaired haemopoietic response to EPO ?

The large multicentre trials of EPO in the United States of America and Europe indicate that 95-98% of patients treated will respond [159]. Nevertheless, there is undoubtedly a small proportion of patients who have either no response or a grossly inadequate one. Even though some of these patients will respond to a much higher dose of EPO, a precipitating cause should first be sought (Table 12). The most common problem is undoubtedly an inadequate supply of available iron, as discussed earlier. Other forms of haematinic deficiency, such as vitamin B_{12} or folate, are less common and should have been excluded before starting treatment. Aluminium toxicity is another cause of erythropoietin resistance, but results from a recent European multicentre trial showed that this has to be severe before haemopoiesis is inhibited [250]. High parathyroid hormone levels inhibit erythropoiesis *in vitro* [24], but the clinical relevance of these findings remains controversial [250,251,252]. Infection, either acute or chronic, as well as occult malignancy are potent suppressors of erythropoietic activity, and several cases have been reported in which this has occurred (see Chapter 2; Fig. 11) [90].

All the above causes of erythropoietin resistance represent examples of an inadequate haemopoietic response. The other possibility is that erythropoietin is effective in initiating enhanced erythropoietic activity, but that there is also increased red cell loss, either through haemolysis or blood loss. A clue to this may be an enhanced reticulocyte response to EPO which is not reflected in any change in the haemoglobin concentration.

Thus, patients showing a poor response to EPO, or loss of a previous response, require investigation for an underlying cause. Haematinic deficiency, aluminium toxicity, haemolysis, and blood loss can be relatively easily excluded, although occult infection or malignancy may prove more difficult. It may be possible to override some of these causes of erythropoietin resistance with a higher dose of EPO, but the importance of excluding them should not be disregarded.

Potential complications

A number of possible complications of EPO therapy have been described, including hypertension [90,91,92,159,173], thrombosis of the arteriovenous fistula [90,92,93,174,175], flu-like symptoms [90,92,174], and increased pre-dialysis plasma potassium concentrations [91,92]. Hypertension occurs the most frequently, and is potentially the most serious consequence of this treatment, occasionally resulting in seizures or encephalopathy. It has been suggested that this is more common in patients with a previous history of hypertension [92], but a large multicentre trial showed no greater risk of a rise in blood pressure between those previously hypertensive and those who were originally normotensive [253]. It is therefore essential that arterial pressure is well-controlled before commencing EPO therapy and that the patient is monitored frequently. Although any increase in blood pressure can usually be controlled by hypotensive agents [253], a rise in blood pressure may be an indication for reducing the dose of EPO with a consequent reduction in haemoglobin concentration. In other patients blood pressure control may be facilitated by aiming for a lower target haemoglobin concentration.

Thrombosis of the arteriovenous fistula was of some concern in the early studies of EPO treatment [90,92,93], and a recent placebo-controlled multicentre trial suggested that there is a significantly increased risk of this complication developing in patients receiving the hormone [175]. Whether this is exacerbated by the increase in blood viscosity (see Chapter 4) [173,185,187], changes in platelet function [206,207,208,209], or a reduction in protein C and protein S levels (see Chapter 5) [210] requires further elucidation.

Flu-like symptoms have been recorded in a few patients early in the course of EPO therapy [90,92,174], but these are of no consequence and disappear even if treatment is continued. Genuine intolerance to erythropoietin sufficient to warrant stopping the hormone is rare. To date, there have been no reports of antibody formation [254].

Some haemodialysis patients are noted to have higher pre-dialysis potassium and phosphate levels with a rising haematocrit [91,92]. In a few individuals this may be

due to increased dietary intake resulting from the general improvement in well-being. However, it is possible that dialyser potassium clearance is lower with higher haemoglobin concentrations [255]. Thus, dietary guidelines should be reinforced for all patients starting EPO treatment.

Table 12 Potential causes of resistance to erythropoietin therapy

1. DECREASED RED CELL PRODUCTION

(i) Iron deficiency

(ii) B_{12}/folate deficiency

(iii) Aluminium toxicity

(iv) Hyperparathyroidism/marrow fibrosis

(v) Infection - acute

- chronic

- occult

(vi) Malignancy - ? occult

(vii) Poor absorption of erythropoietin (if given subcutaneously)

(viii) Marrow dysfunction

(ix) Red cell enzyme abnormalities, e.g. pyruvate kinase deficiency

2. DECREASED RED CELL SURVIVAL

(i) Blood loss - dialysis

- other ? gastrointestinal tract, ? occult

(ii) Haemolysis

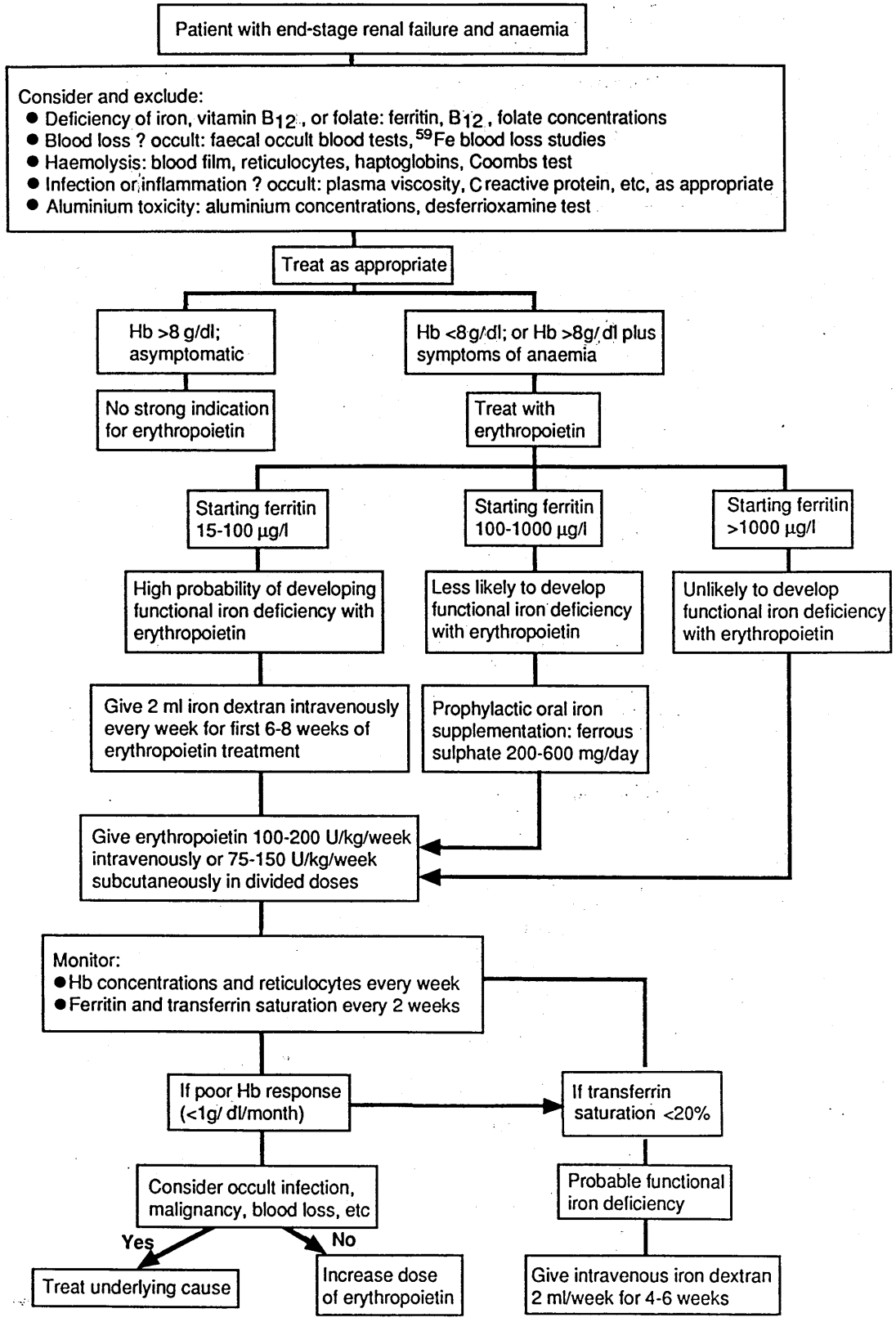


Fig. 46 Clinical algorithm for treating renal anaemia with recombinant human erythropoietin therapy.

11. CONCLUSIONS

The last decade has been an exciting one not only for investigators working in the field of erythropoietin, but also for the patients who have benefitted from this revolutionary new treatment. The pace of progress has been rapid, with the journey from isolation of the human EPO gene, to its cloning, to animal testing, and to clinical use proceeding with remarkable speed and a notable lack of serious problems. The gene for human EPO was found in 1983 [53], the recombinant hormone was tested in mice and dogs by the Amgen Corporation in 1984-1985 [54], and the first clinical trials in human haemodialysis patients began in December 1985 [91].

Recombinant human erythropoietin (or epoetin, as it has subsequently been named) became licensed for the treatment of renal anaemia by the Food and Drug Administration (FDA) in the USA on 1st June 1989; several European countries followed suit, and the drug was finally licensed for use in the UK in May 1990. During this period, many thousands of renal patients worldwide have been treated, with striking improvements in their quality of life and sense of well-being. The secondary consequences of a rise in haemoglobin concentration have gradually emerged, and much of the work reported in this thesis has advanced this knowledge. In this respect, the findings from the study of long-term cardiorespiratory effects of EPO therapy published in the Lancet in March 1990 (and reported in Chapter 3 of this thesis) gave the first objective evidence of improved cardiac function, and the reversal of myocardial ischaemia and regression of left ventricular hypertrophy following treatment with EPO suggests that we might yet anticipate a long-term improvement in morbidity and mortality in treated dialysis patients.

The pharmacokinetic results detailed in Chapter 1 suggested that the subcutaneous route might be the most practical one for administering EPO to both haemodialysis and CAPD patients. Since then, numerous studies (including those documented in Chapter 2) have reported on the efficacy of SC-administered EPO therapy [114,115,116,117,118,119,127,128,129], and there is accumulating evidence that lower doses may be used when given by this route than when given IV. In addition, the

pharmacokinetic study cast serious doubt on the practicability of using IP-administered EPO in CAPD patients; apart from a preliminary report in 5 patients treated with IP EPO [103], this route of administration has since become obsolete.

Concern still exists over the possible development of hypertensive and thrombotic complications in dialysis patients treated with EPO. While the pathogenesis of these side-effects remains unclear, the detailed studies on blood viscosity and coagulation parameters reported in Chapters 4 and 5 yielded useful information regarding possible contributory factors. In this respect, it has been interesting to learn that recent studies of rheumatoid arthritis patients treated with EPO [256,257,258] have not witnessed the hypertensive problems with EPO that have been evident in the studies on renal patients.

The difficulties with ^{125}I-labelled EPO reported in Chapter 6 were not predicted and, to some extent, hampered progress in this field. There is still remarkable ignorance surrounding the organ distribution and metabolic fate of either endogenous or recombinant EPO, and further studies are required.

What then of the future? Attentions have more recently turned to focus on other applications of EPO therapy. Preliminary reports suggest that it may have a role in the management of anaemic conditions other than that associated with end-stage renal disease. These include the anaemias associated with AIDS therapy [259,260], malignant disease [261] and cancer chemotherapy [262], prematurity [263,264,265], rheumatoid arthritis [256,257,258], sickle-cell anaemia [266,267], and myelodysplastic syndrome [268,269], in addition to increasing supplies of autologous blood for peri-operative use during surgery [270,271]. Of greater concern is the alleged increasing use, or rather abuse, of EPO in sports medicine. By boosting "super-haemoglobin" levels in athletes, EPO has the same effect as high-altitude training in increasing the exercise capacity and stamina of marathon runners and long-distance cyclists. Detection of this abuse is extremely difficult owing to the identical structural and immunological properties of the recombinant product compared with the native endogenous hormone.

With the exception of this potential for abuse in sports medicine, however, the future for EPO looks bright. It has already transformed the lives of several thousands of individuals, and promises to yield further exciting prospects for many years to come.

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