TUBERCULOSIS, ANAEMIA and ERYTHROPOIETIN

A study evaluating the role of erythropoietin in the pathophysiology of the anaemia of tuberculosis.

OSMAN EBRAHIM
BSc(Hons), MBChB, MSc(Med)

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DECLARATION

This is to certify that this thesis is my own work and has not been presented for a degree at any other University. Part of this work was carried out in the laboratory of Dr P Ratcliffe, Institute of Molecular Medicine, University of Oxford. He has granted permission for my work to be incorporated into this thesis.

This ----- Day of July 1997.

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DEDICATION

This work is dedicated to my wife, ***Shamain****, for her love, support and encouragement over the years and for her patience and sacrifice during the time it took to complete this thesis.*

The clinical and experimental studies described in this thesis were carried out over a period of three years, one year of which was spent as a Research Fellow at the Institute of Molecular Medicine, University of Oxford. The work has been published in the European Journal of Haematology and Biochemica Biophysica Acta. This work was presented at the Medicine Research Day, University of Cape Town Medical School and was awarded the prestigious Bernard Pimstone Prize. It took a further two years to complete the work as I became seriously ill during the study and on recovery worked as a Medical Registrar at a busy teaching hospital which left little time to write up the thesis.

Abstract

Although it is more than 100 years since Robert Koch discovered the tubercle bacillus, and more than 40 years since effective chemotherapy became available, tuberculosis remains a major cause of morbidity and mortality in the world to-day. A major contributor to the morbidity is anaemia. This anaemia falls under the classification of anaemia of chronic disorders, the pathogenesis of which has not been fully elucidated. With the advent of recombinant human erythropoietin (Epo), it has become evident that a blunted Epo response to the anaemia plays a major role. The mechanism (s) involved still have to be elucidated.

The aims of this study were to evaluate serum Epo levels and iron parameters in anaemic patients with active pulmonary tuberculosis (PTB) and investigate the effect of tumour necrosis factor alpha (TNFα) produced by activated macrophages in PTB patients on Epo production *in vitro*. Furthermore the mechanism involved in Epo gene expression was investigated.

Haematological and biochemical parameters (including serum iron and Epo) were studied prospectively in four groups each of 10 subjects. Group I comprised newly diagnosed non-pregnant individuals with pulmonary tuberculosis (PTB), haemoglobin below 110 g/L, and having no apparent dissemination or other associated systemic illnesses. Group II were age and sex matched PTB patients with haemoglobin levels greater than 130 g/L. Group III consisted of otherwise healthy people with demonstrated absolute iron deficiency anaemia with haemoglobin corresponding to those in Group I. Group IV consisted of 10 healthy non-anaemic volunteers. For matching degrees of anaemia, the serum Epo was significantly lower in Group I than in Group III patients. In PTB, therefore, the Epo response to anaemia is attenuated. With anti-tuberculous therapy there was a significant increase in the Hb and serum iron levels in the Group I patients which correlated with a fall in the levels of the inflammatory marker C-reactive protein (CRP). This argues for the degree of inflammation being casually related to the anaemia.

To further investigate the effect of inflammation on Epo production, blood samples were collected from individuals in Groups I, II and III and the peripheral blood mononuclear cells (PBMC) were assayed for the cytokine TNFα. The incubation of supernatant fractions (SNF) of the anaemic PTB group with HepG2 cells resulted in a marked inhibition of Epo production by these cells.. Dose response studies showed that increasing concentration of SNF resulted in a progressive reduction in Epo production, which could be reversed by the presence of anti-TNFα antibodies in the medium. Thus TNFα is capable of inhibiting Epo production and may play a role in the blunted Epo response to anaemia seen in patients with PTB.

In order to characterize the mechanisms involved in oxygen sensing, the murine Epo gene was studied to define the sequences within the enhancer involved in oxygen sensing and Epo gene expression. To this end, transfection experiments of deleted, mutated and re-iterated enhancer sequences located 3' to the poly (A) signal sequence were carried out in HepG2 cells and in the non-Epo producing lung fibroblastoid cell line a23. Transcription factor binding to the enhancer was investigated by DNA footprint analysis and revealed that at least three sites within a 96 nucleotide sequence of the Epo enhancer were critical. Oxygen regulated operation was dependent on sites within the first 25 nucleotides. In both HepG2 and a23 cell lines the same two critical sites in the 5' region of the enhancer were necessary for function. Sequences located 3' to this region modulated enhancer function but did not themselves convey oxygen regulated operation.

This study has contributed to the understanding of the pathophysiology of the anaemia of tuberculosis in that in this disease, TNFα released from activated macrophages was capable of inhibiting Epo production *in vitro*. This may explain the attenuated Epo response to anaemia in PTB patients. Furthermore three critical sites on the Epo enhancer were shown to be essential for oxygen sensing and Epo gene expression. It can be postulated, therefore, that TNFα may disrupt the interaction of transcription factors with critical sites of oxygen sensing on the enhancer and prevent the increased production of Epo. Further work is required to clarify this postulate.

Chapter 1

LITERATURE REVIEW

1.1 Introduction

Inflammation may be defined as the organism's response to injury irrespective of the aetiology. Although this is to protect the host, certain negative consequences may ensue. Mild or moderate anaemia, for example, frequently accompanies such diverse disorders as tuberculosis, rheumatoid arthritis, Crohn's disease and cancer. This is probably the commonest form of anaemia encountered in hospital practice and has characteristic morphologic, biochemical and kinetic alterations and is termed anaemia of chronic disorders.

Haematological changes associated with pulmonary tuberculosis have been incompletely investigated in that only the prevalence and severity have been documented and no work has been carried out on the pathophysiology of the disease.

The purpose of this review is to encompass a spectrum of clinical and scientific publications with respect to the epidemiology, immunopathogenesis of tuberculosis and report on studies carried out to elucidate the pathophysiology of anaemia of chronic disorders and particularly the role of erythropoietin in this regard.

1.2 TUBERCULOSIS

1.2.1. Epidemiology:

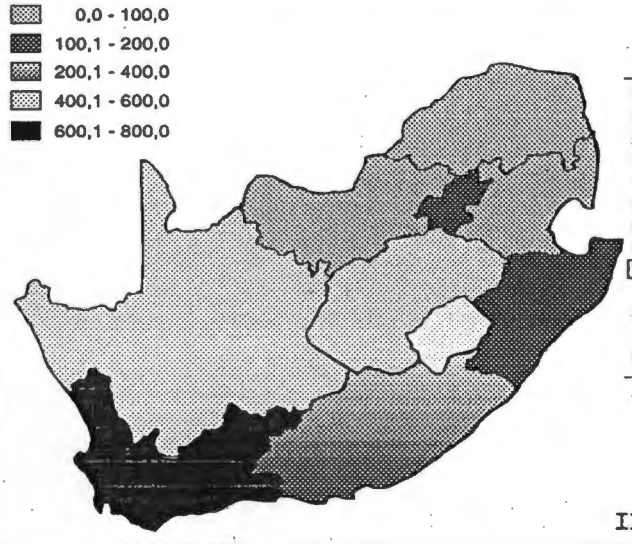
Globally the incidence of tuberculosis (TB) is rising rapidly : 1.7 billion people are infected with Mycobacterium tuberculosis world-wide, with 8 million new cases of the disease and almost 3 million deaths occurring annually (1).

This has led the World Health Organization (WHO) to take the unprecedented step of declaring TB a global emergency. This is in itself an ominous statement and, have suggested that four major elements have played a role in the creation of the global TB emergency (2). These included the following :-

- Poor quality TB control programmes which fail to cure but keep infectious patients alive, thus increasing the infectious pool in the community.
- The HIV epidemic which has the propensity of worsening the situation dramatically
- Socioeconomic trends of the past few decades, including increased immigration, influx of refugees and economic recession, resulting in cuts in public spending with deterioration of public health services and increasing poverty. The relative importance of these elements obviously differs in industrialised and developing countries.
- Demographic changes (decreased child mortality with a consequent increase in the number of people over 15 years of age, many of whom were infected with TB as children) result in more cases of TB even if the rate at which TB occurs remain stable.

In South Africa, as in other sub-Saharan African States, poor quality TB control programmes, HIV infection and demographic changes are important factors in the rapid rise in tuberculosis (3). The Western Cape, the area where this study was carried out, has the highest incidence of TB in the whole of South Africa, at 702.6/100,000 (Figure 1.1) (4). Furthermore ethnic differences exist in the incidence of TB, with the coloured population having an incidence rate which is progressively increasing compared to the other population groups (Table 1.1). However, there are no South African data which indicate that the coloured population have increased genetic susceptibility to activation of latent TB infection. Thus the cause for the geographic and ethnic distribution of TB in South Africa is as yet unknown.

Figure 1.1: Demographic distribution of tuberculosis in South Africa as on 25/01/95



Province	Cases	Population	IR
Eastern Cape	17870	6368655	280,59
Eastern Transvaal	2401	2853212	84,15
Gauteng	12969	6757225	191,93
KwaZulu/Natal	9695	8409594	115,28
Northern Cape	3130	750271	417,18
Northern Transvaal	2654	4993708	53,15
North West	2731	3292393	82,95
Orange Free State	12990	2751972	472,03
Western Cape	24858	3537980	702,60
South Africa	89298	39715010	224,85
Outside South Africa	439		
Total	89737		

IR = Incidence Rate/100000

Adapted from Tuberculosis Update (from ref 4)

Table 1.1 The incidence rate (IR) per 100 000 population of tuberculosis per population group, South Africa, 1990 - 1994 (as on 23/01/95)

Population Group	1990			1991			1992			1993		
	Cases	Population	IR	Cases	Population	IR	Cases	Population	IR	Cases	Population	IR
Asian	580	975994	59,43	547	990994	55,20	558	1005994	55,47	519	1021994	50,78
Black	59093	27931168	211,57	54481	28664884	190,06	58205	29407593	197,93	62293	30154302	206,58
Coloured	19604	3245745	603,99	21501	3296667	652,20	22172	3346586	662,53	24201	3396505	712,53
White	882	5037947	17,51	810	5073300	15,97	967	5108254	18,93	964	5142209	18,75
Unknown	49			154			397			1321		
Total	80208	37190854	215,67	77493	38025845	203,79	82299	38868427	211,74	89298	39715010	224,85

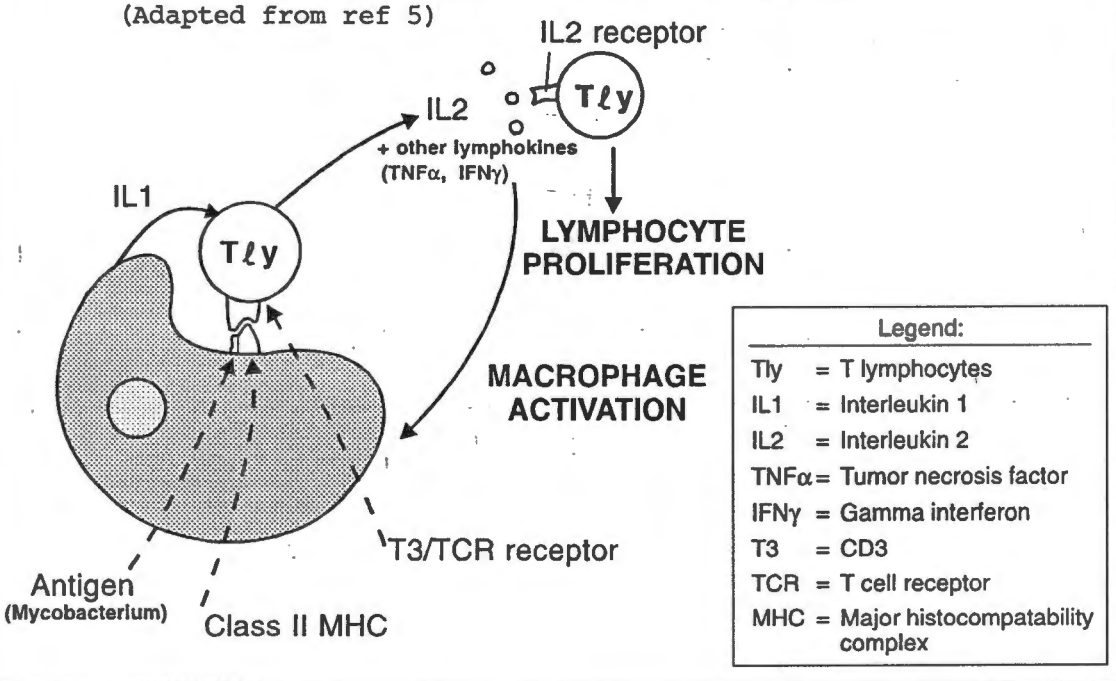
Adapted from Tuberculosis Update (from ref 4)

1.2.2 Immunopathogenesis of Tuberculosis :

On entry into the immunocompetent body the tubercle bacilli are processed by macrophages and then expressed on their cell membranes in conjunction with the class II major histocompatibility complex antigen (HLA-DR). This complex binds with the T-Cell antigen receptor (TCR) in T lymphocytes and, together with a second signal of interleukin-1 (IL-1) secreted from the macrophage generates the specific immune response (Figure 1.2) (5). In this way the T-lymphocytes become 'activated' dividing, expressing several surface receptors and secreting a variety of immune modulators called cytokines. The cytokines attract macrophages and lymphocytes to the site of infection, and these macrophages become 'activated', enlarging and becoming more efficient at phagocytosis and killing. Some macrophages are transformed into more specialised forms eg. epitheloid cells (more secretory than phagocytic) and multi-nucleated giant cells. The aggregation of these macrophages and lymphocytes results in the characteristic granuloma of tuberculous infection.

An individual newly infected with M.tuberculosis only has a 2 - 5% risk of developing active disease following primary infection and an approximate 10% lifetime risk of developing post-primary tuberculous disease (6). Thus, there is an enormous gap between infection on the one hand and disease on the other, and the vast majority, up to 98%, have no difficulty in mounting a highly effective protective immune response that completely suppresses and perhaps even eradicates this infection.

Figure 1.2: Specific acquired immunity: Macrophage together with MHC II presents the antigen (mycobacterium) to the T lymphocytes. This results in release of various cytokines causing lymphocyte proliferation and further macrophage activation.
(Adapted from ref 5)

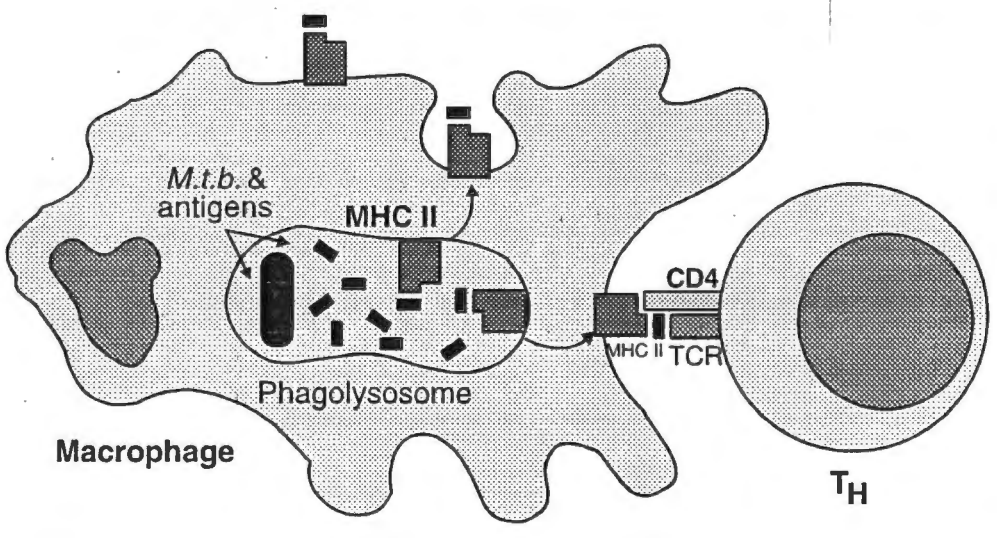


Recently, researches have investigated the reasons why most people contain tuberculous infection and why others develop disease. Mycobacteria causes two different immune responses which were previously thought to be inextricably linked: delayed hypersensitivity with tissue damage (Koch phenomenon) and protective cellular immunity. Recent immunological studies *in vitro* (7), have shown that there are two distinct responses which can be separated, giving important clues to the variability in host responses to tuberculosis.

Once an infection has been established, resistance to the progression from infection to disease requires a full-blown cell-medicated immune response, which depends on T-Cells. The T-helper subset of lymphocytes, bearing the CD4 (cluster differentiation antigen) surface marker, recognise antigen that is presented by antigen-presenting cells such as macrophages in the context of major histocompatibility (MHC) class II

molecules (Figure 1.3) (8). Once activated the T-helper cells response is polarised between the so-called Th 1 and Th 2 divisions, which are primarily distinguished from one another in their profiles of secreted cytokines (Figure 1.4). The distinction between T-helper cells of Th 1 and Th 2 types is a recent and important advance in the understanding of protective immunity in infections (9). The Th 1 cells secretes in particular interferon gamma (IFNγ) which contributes to the developments of cell mediated immunity with activation of macrophages and enhancement of cytotoxic cells. On the other hand, the Th 2 cell secrete predominantly interleukin 4 (IL-4) which antagonises most of the effects of IFNγ and this inhibits the cell mediated response. It is likely that a shift from a Th 1 to Th 2 response in TB will lead to a failure of protective immunity and may be a factor in progressive tuberculous disease (10).

Figure 1.3: Major histocompatability (MHC) class II processing and presentation *of M.t.b* antigens. Antigens derived from bacilli resident in phagosomes are processed and associate with MHC class II molecules. These are then transported to the cell surface where they are "read" by T cells of the CD4+ T helper (T_H) suset. The T cell receptor (TCR) in association with CD4 binds to the MHC II-antigen complex, thereby stimulating the T_H cell.



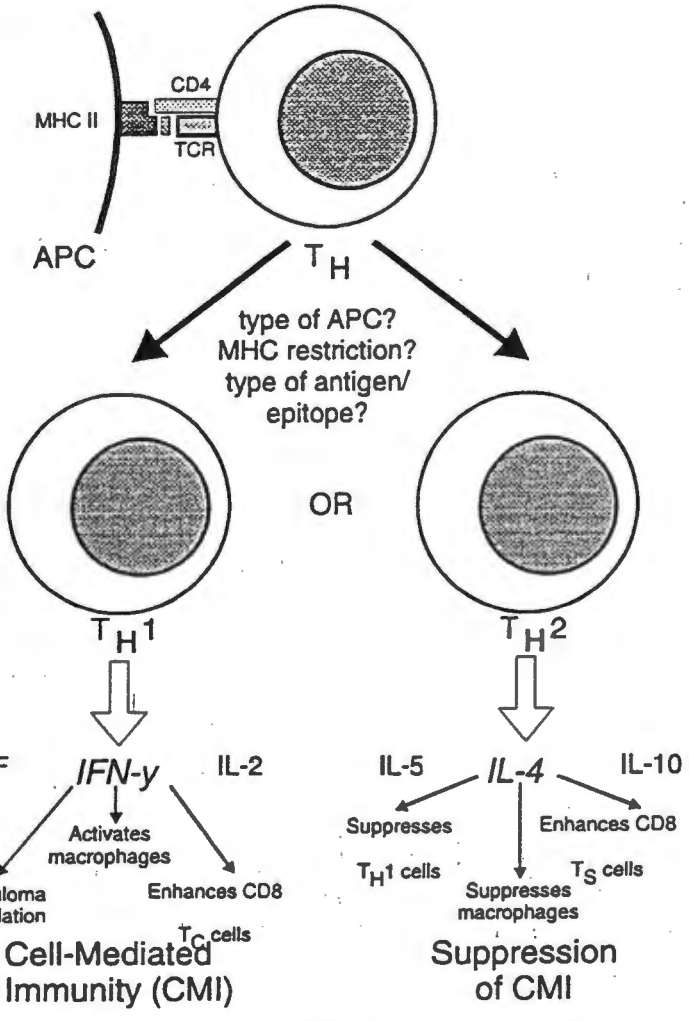
Adapted from Tuberculosis/HIV - Proceedings of an update conference. University of Stellenbosch, Cape Town, 1995.

1.2.3 Tumour Necrosis Factor alpha and Tuberculosis :

Tumour necrosis factor alpha (TFNα) also referred to as cachectin was discovered during studies of rabbits infected with Trypanosoma brucei that exhibited extreme weight loss, depletion of body mass and hypertriglyceridaemia. Rouzer and Cerami (11) found that elevated serum levels of triglycerides were caused by suppression of lipoprotein lipase (LPL). Subsequent work showed that mice infected with endotoxin produced a transferable serum factor that causes LPL suppression (12). This factor was successfully isolated by Beutler and others (13), and the name "cachectin" was given because of its suspected role as a mediator of cachexia. The role of TFNα in tuberculosis is paradoxical because although there is much evidence for a protective role, there is much evidence that it plays a part in tissue damage that characterises human disease.

Studies in the mouse, using gene knockout or neutralising antibodies, indicate that protection from M.Tb is mediated by a type 1 response. That is, it requires a Th 1 pattern of helper activity with secretion of IFNγ, accompanied by MHC I restricted functions, presumably CD8+ cytotoxic T cells (14). Together these cell types can activate macrophages and perhaps kill infected cells that are failing to eliminate the bacilli. Experiments with neutralising antibodies indicate that TNFα is an essential component of the process (15). This may reflect the ability of the cytokine to trigger further killing mechanisms, such as nitric oxide production, in macrophages previously activated by IFNγ (16) or it may be something to do with the putative role of TNFα in the organisation of granulomata (15).

Figure 1.4: Polarisation of the T helper (T_H) cell response.



Adapted from Tuberculosis and HIV/AIDS - Proceedings of an Update Conference. University of Stellenbosch, Cape Town, 1995.

On the other hand, TNFα plays a role in the tissue damaging immunopathology that is characteristic of TB. The fever, weight loss and tissue injury that characterises advancedTB may be attributable in part to TNFα (17). This is probably due to stimulation of the release of collagenase from human synovial cells and dermal fibroblasts, by TNFα, which possibly contributes to tissue destruction and remodelling(18).

A potential resolution of this paradox emerged when Hernandez-Pando and Rook (19) showed that murine spleen cells when immunised with a dose of mycobacterium that produced a 'pure' Th 1 response, TNFα acted as an additional macrophage activating factor without precipitating necrosis. However a dose of mycobacterial immunogen that produced a mixed Th 1 and Th 2 response caused tissue damage. This mixed pattern is characteristic of tuberculosis, and of the late stage of many chronic infections where elimination of the infecting organism is failing and chronic tissue damage is seen. It has been suggested that immunological manipulations which eliminate the Th 2 component, and boost the Th 1 response may allow the immune system to play a more useful role in treatment of the disease (20).

1.2.4 Anaemia of Tuberculosis:

Haematological changes associated with pulmonary tuberculosis (PTB) have been incompletely investigated in that only the prevalence and severity have been reported and no work has yet been carried out to elucidate the pathophysiology of the anaemia associated with the disease.

Baynes *et al* (21) evaluated 59 patients with active PTB in terms of haematological indices, iron related measurements and markers of inflammation. They found that anaemia was present in 76 % of patients with active PTB and in these patients the serum ferritin was raised which correlated with the degree of inflammation as measured by the C-reactive protein. In addition, there was a good correlation between serum ferritin and non-haem iron in the marrow.

They concluded that although iron is diverted to the reticuloendothelial system, in active PTB, there was no evidence to suggest that the anaemia was secondary to iron-deficient erythropoiesis as the percentage saturation of iron was 30%.

Morris *et al* (22) studied the extent and severity of haematological and biochemical abnormalities in 265 patients with PTB. They showed that anaemia (Hb <11 g/dl, normochromic and normocytic) was present in 60% of patients and more frequently in males than in females. Serum ferritin, vitamin B12 levels were elevated in 57% of subjects, whilst serum and red cell folic acid were within normal limits in the majority of patients. With anti-tuberculous therapy, there was a significant increase in the mean haemoglobin level from 11 g/dl to 12.3 g/dl at three months when sputa became acid fast bacilli negative. Important biochemical changes noted were hyponatraemia and hypoalbuminaemia.

From these studies it can be seen that the anaemia of PTB can be classified as anaemia of chronic disorders in that the anaemia is mild, with a raised serum ferritin. In order to understand possible pathophysiological mechanisms of this anaemia studies involving other examples of anaemia of chronic disorders will be reviewed.

1.3. ANAEMIA OF CHRONIC DISORDERS

1.3.1 Description of the anaemia:

In his classic review in 1966, Cartwright (23) defined the anaemia of chronic disorders (ACD) as follows:

"The anaemia is usually mild in the degree, not progressive in the severity and is characterised by a low plasma iron, decreased total iron binding capacity, decreased saturation of transferrin with iron, decreased bone marrow sideroblasts, normal or increased reticuloendothelial iron, increased plasma copper and increased free erythrocyte protoporphyrin.".

Although the anaemia is characteristically normochromic, hypochromia and microcytosis are seen when the serum iron concentration and more particularly the transferrin saturation are reduced (24).

Iron deficient erythropoiesis occurs when the percentage saturation of transferrin falls below 16% but this occurs uncommonly in ACD (25). Furthermore, with the fall in serum iron and transferrin saturation there is a marked increase in serum ferritin - a finding which is the most convenient way of distinguishing ACD from iron deficiency anaemia (26).

Other biochemical changes occur in ACD, including synthesis of new proteins such as C-reactive protein (27), decreased levels of albumin and transferrin (28) and an augmented synthesis of fibrinogen (29), which may account for the accelerated erythrocyte sedimentation rate (ESR)(23). Bone marrow morphology is generally normal although there may be a degree of erythroid hypoplasia and defective red cell haemoglobinization. The most characteristic finding on bone marrow examination, however, is a reduction in the number of sideroblasts while the reticulo-endothelial system (RES) iron deposits are normal or even increased (30).

1.3.2 Pathogenesis:

Early investigators proposed that three processes were involved in the production of this anaemia:

- a modest shortening in red cell survival
- disturbance in iron metabolism
- impaired erythropoietin (Epo) production

1. Haemolysis:

The abnormal processes resulting in reduced erythrocyte survival have not been clearly defined. Two mechanisms by which this might occur have been proposed:

Cartwright (23) favoured the hypothesis that the reticuloendothelial system (RES) became hyperactive in removal of red cells from the circulation. This increased phagocytic activity of the RES was presumably related to macrophage activation (31), the activation process being partly related to lymphokines released by immunologically committed lymphocytes (32). These changes are associated with greatly increased clearance rates for erythrocytes sensitised with antibodies (33). Antibodies coating erythrocytes in amounts too small to produce changes in red cell survival in normal animals lead to dramatic increased in red cell destruction in animals with activated macrophages. Similarly, enhanced removal of red cells midly damaged with heat has been observed in animals given endotoxin (34). However, these studies employed damaged or sensitised red cells and uncertainty remains as to the applicability of these findings to normal cells.

When fever and inflammation were induced in rabbits by injections of bacterial endotoxin, excess destruction of red cells occurred (35). Although the injections might have produced their effects indirectly, for example, by stimulation of the macrophage system, experiments in heating chambers showed that the destruction of red cells was primarily due to increased temperature and erythrocyte damage preferentially affected the older segment of the red cell population (36). These studies support the hypothesis that fever may damage the membrane of older erythrocytes and that such damage may play a role in the excessive destruction of red cells observed in ACD. However, reticulocyte counts are variable in ACD, thus there is universal agreement that the modest haemolysis seen in ACD is not a major contributing factor to the anaemia (37).

2. Disturbances in Iron Metabolism:

The characteristic feature of ACD is hypoferraemia despite iron stores that are either normal or raised. Cartwright (23) showed that there was a negative correlation between the level of serum iron and disease activity. Measures to suppress the inflammatory process in chronic disease have been followed by a sharp rise in serum iron levels (38,39).

The fact that iron stores may be plentiful even if erythrocytes showed signs of iron deficient erythropoiesis, lead to the postulate by Cartwright (40) that this was due to a block in the release of iron from the RES to the erythron. In a study of dogs, Fillet et al (41) found that iron was released from he RES in two phases, a rapid one taking place within hours, and a slower one taking place over several days. Furthermore, they showed that acute inflammation was associated with abolition of the first rapid phase, thus providing some support for Cartwright's postulate of blocked RES iron release.

In a study in man, Bentley et al (42) could not demonstrate this dual release mechanism and found no evidence of a blocked RES iron release in nine patients with rheumatoid arthritis. Similarly Baynes et al (21) studying patients with active pulmonary tuberculosis showed that although iron is diverted to the RES, there was no evidence to suggest that the anaemia was due to iron deficient erythropoiesis, as the percentage saturation of iron in the anaemic pulmonary tuberculosis group was 30%. These investigators postulated therefore, that the plentiful iron stores in ACD were not due to blocked RES iron release but were secondary to diminished use of iron due to a primary defect in erythropoiesis.

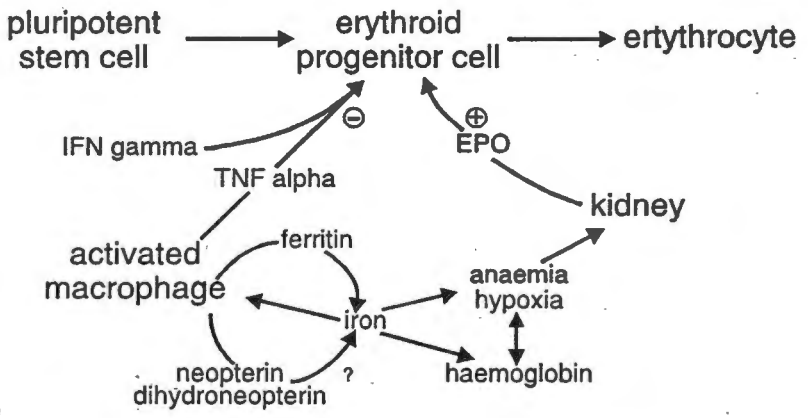
Lactoferrin is an iron binding protein found in large quantities in the secondary granules of neutrophilic granulocytes and is released into the plasma during an inflammatory process. Its affinity for iron is greater than that of transferrin, especially at low pH (43), and it is taken up rapidly by macrophages (44).

Bacteria require significant quantities of iron for metabolism and replication (45). The movement of iron out of plasma augments the resistance of the host to invading bacteria (46). Studies carried out by Van Snick et al (47,48,49) suggested that lactoferrin might bind iron in extracellular fluid and transfer it to ferritin, predominantly in RE cells. This would be entirely in keeping with its postulated antimicrobial activity. Thus the theory is interesting, but still unproven. Furthermore, even if it may apply to the anaemia of acute bacterial infection, in which plasma lactoferrin is high (50), this mechanism could hardly explain the hypoferraemia in rheumatoid arthritis, in which plasma lactoferrin is normal (51).

Serum ferritin increases during inflammation. This may be due to an acute phase response (52) or to a leak from cells whose membranes have been damaged by inflammation (53,54). Serum ferritin is glycosylated whereas intracellular ferritin is not (55). Thus it has been resolved that the raised serum ferritin seen in inflammation is the result of increased synthesis and not leakage from damaged cells (56).

Furthermore, it has been shown that the increased synthesis of ferritin by the liver in animals with inflammation occurs before the fall in serum iron (57). Macrophages have also been shown to synthesise ferritin in response to inflammation and with the increased synthesis there was an increased uptake of iron by these cells (Figure 1.5) (58).

Figure 1.5: Influence of activated macrophages and specific cytokines, interferon (IFN) gamma and tumour-necrosis factor (TNF) alpha. on iron metabolism and haematopoiesis. (Adapted from ref 58).



The impaired iron mobilisation may also result from effects of cytokines. Denz et al (59) have reported a correlation between the immune activation marker neopterin and increasing ferritin levels in patients with malignancies, suggesting a role of immune activation in altered iron metabolism. Rogers et al (60) showed that interleukin-1 (IL-1) increased translation of ferritin mRNA and this additional ferritin could act as a trap for iron that might otherwise be available for erythropoiesis. Moldawer et al (61) and Alvarez-Hernandez et al (62), reported that rodents injected with recombinant TNF develop a hypoferraemic anaemia associated with impaired iron release and incorporation into erythrocytes.

Studies of the biology of the cellular transferrin receptors (TfR) yielded additional insight into the mechanisms by which inflammatory response may alter iron metabolism. Graziadei and colleagues (63) reported that acute phase reactants such as α-1-antitrypsin inhibited erythropoiesis by impairing transferrin binding to TfR and subsequent internalisation of TfR-transferrin complex. Study of the cellular TfR has also provided clinically diagnostic assistance in ACD. TfR are increased in iron deficiency anaemia but is normal in ACD, providing a useful method to distinguish these often confused syndromes (64).

3. Relative Deficiency of Erythropoietin:

The recognised role of Epo in the pathogenesis of ACD is becoming increasingly evident. However, before the studies are reviewed, physiological and biochemical aspects of the hormone will be discussed.

1.4 ERYTHROPOIETIN

1.4.1 Historical Background:

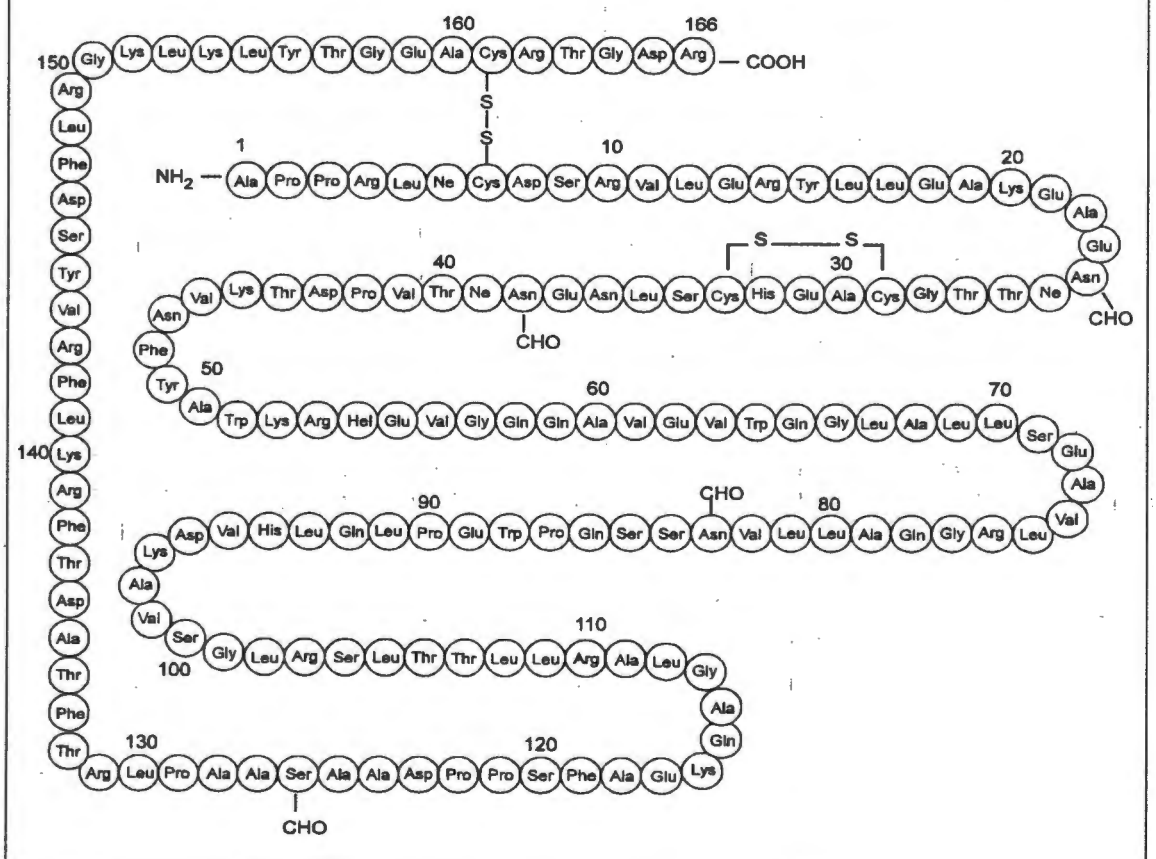
In 1906 Carnot & DeFlandre (65) gave the name "hemopoietine" to a humoral factor that was thought to control red blood cell production. Later this hormone was more appropriately named "erythropoietin", implying restricted action of the humoral factor on red blood cell production, by Bonsdorff and Jalavisto (66). Erslev in 1953 (67) provided definitive evidence of the existence of erythropoietin when he demonstrated the occurrence of reticulocytosis in rabits infused with large volumes of plasma from severely anaemic donor rabbits. In fact, Erslev predicted then, the potential therapeutic value of erythropoietic factor once available in purified form. Shortly thereafter, other investigators succeeded in demonstrating erythropoietic activity in plasma (68,69) and urine from anaemic rabbits (70).

1.4.2 Structure of Erythropoietin:

The human urinary Epo, purified to apparent homogeneity by Miyake et al in 1977 (71), consisted of two fractions (α and β) of similar biological activity, molecular mass, and aminoacid sequence but different electrophoretic mobility and carbohydrate composition.The native molecular mass of the polypeptide chain is 18 kDa (72), while the glycosylated form has a molecular mass of 30 kDa (73) Human urinary and recombinant DNA-derived Epo are identical with regard to amino acid sequence, position of disulfide bonds, glycosylation sites , and secondary structure (73). In its physiologically active form, Epo is made up of 165 aminoacids with two disulphide bonds (Figure 1.6) (72).

The disulfide bridges are formed by cysteines at position 7 and 161 and 29 and 33. Furthermore, Epo is made up of 40% carbohydrate containing high amount of sialic acid (74). Minimal loss of sialic acid from the carbohydrate moiety results in almost complete loss of biological activity in *vivo* as measured by the ex-hypoxic mouse assay (75). The carbohydrate residues although not important for its physiological function, prevent its rapid removal from the circulation (76).

Figure 1.6: The primary amino acid sequence of human erythropoietin. The sequence is folded arbitrarily for illustration. Two disulphide bonds link Cys 7 with Cys 161 Cys 29 with Cys 33. The three N-linked glycosylation sites occur at aspartyl residues 24, 38 and 83. One O-linked glycosylation site is present at Ser 126.



(Adapted from Lappin TRJ & Maxwell AP. Chemistry and assays of erythropoietin. In: Jelkmann W, Gross AJ (eds) Erythropoietin 1989).

1.4.3 Sites of Production:

Kidney:

Soon after birth the major site of Epo production changes from the liver to the kidney. Several studies have addressed the question as to which renal cells synthesise the hormone. Immunohistochemical studies with purified antiserum to recombinant human Epo, indicated the tubular cells as the major site of Epo production in hypoxic mouse kidneys (77). *In situ* hybridization data, however, are controversial. Koury et al (78) and Lacombe et al (79) showed Epo messenger RNA is located in the peritubular cells in the cortex and the outer renal medulla of kidneys from anaemic mice. Some of the radiolabelled cells in these studies had an endothelial-like morphology (78) and they contained factor VIII-related antigen (79). Thus peritubular capillary endothelial cells have been considered the most likely candidates for expressing Epo mRNA in the hypoxic murine kidney. In a recent study, however, in which digoxigenin-labelled oligonucleotide probes were employed, *in situ* hybridization for Epo messenger RNA, localized signals in the proximal tubular cells of the inner third of the renal cortex of hypoxic mouse kidney (80).

Liver:

The liver is the primary site of the foetal synthesis of Epo (81,82), and it is also the main extrarenal site of the production of blood-borne Epo in adults (83). The site of production within the liver is not clear. Fried et al (84) considered the bile duct epithelium to be the site of the synthesis of Epo in the liver, because elevated plasma levels of the hormone were measured in rats with bile duct hyperplasia. Immunofluorescent studies located the site of Epo production to the Kupffer cells (85) in extirpated fetal mouse liver. *In situ* hybridization showed that Epo production occured predominantly in centrilobular epithelial cells in the livers of transgenic and normal mice following the induction of anaemia (86).

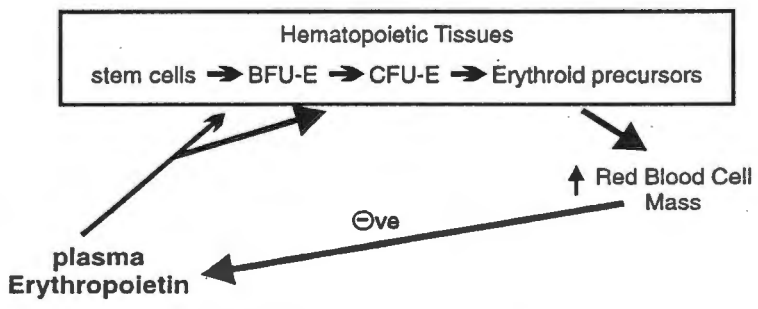
Tissue macrophages:

Murine myeloid and splenic macrophages in culture have been reported to contain erythropoietic activity (87.88). In normal mouse marrow smears, 30% of all macrophages were found to be positive for Epo gene expression by hybridization studies (89). On the basis of these findings, it has been argued that the paracrine release of Epo from macrophages maintains Epo under steady-state conditions, whereas the endocrine production of the hormone in the kidneys and liver is of importance during hypoxic stress (90).

1.4.4 Regulation of Formation of Erythropoietin:

Epo has its predominant effect on the committed erythroid cells defined as the colony-forming unit erythroid (CFU-E)(91). Epo promotes their differentiation and development into mature red blood cells which are then released into the circulation. This process is under negative feedback control (Figure 1.7) (92). The increased proliferation of red blood cells results in the increased red cells mass which 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 (high altitude), anaemia, ischaemia, increased metabolic rate, respiratory disease and toxins affecting the oxygen affinity of haemoglobin.

Figure 1.7: Model for the regulation of erythropoiesis (Adapted from ref 92).

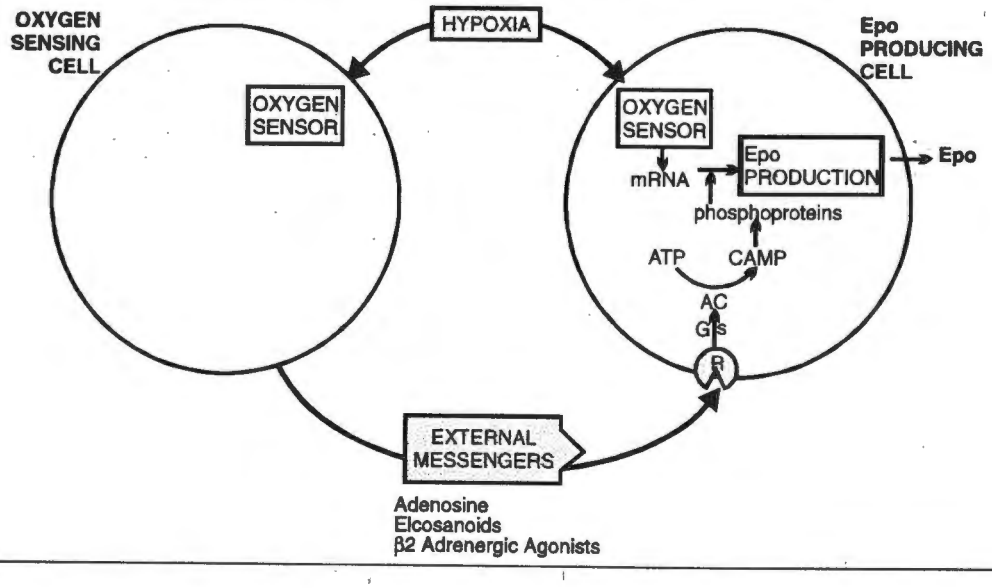


The stimulus for erythropoiesis appears to be oxygen deprivation in a critical renal sensor cell resulting in the initiation of a cascade of events leading to increased synthesis and/or secretion of Epo (93,94). Possible mediators thought to be released during hypoxia and involved in the synthesis and or release of Epo include: adenosine, eicosanoids, oxygen free radicals, cathecolamines and calcium. Apart from adenosine there has been no convincing evidence that any of these substances have any pathophysiological significance. Ohigashi et al (95), using two new adenosine A2 agonists, showed marked increases in Epo production by infusion of the compounds in exhypoxic polycythaemic mice. The compounds also produced significant increases in mean levels of Epo in cloned Epo-producing Hep3B cell line after 18 hours incubation in 1% O_2. More recently, Nagashima and Karasawa (96) determined the effects of adenosine receptor agonist and antagonist on serum Epo concentration in normal and anaemic rats. They concluded that adenosine mediates the Epo production in response to hypoxia in the kidney. The fact that theophylline, a non-specific adenosine antagonist, significantly attenuated the production of Epo in eight patients with erythrocytosis after renal transplantation (97), supported the idea that adenosine may be involved in the transfer of "messages" from the oxygen sensing cells to the Epo producing cells in the kidney (Figure 1.8). Furthermore, this study suggested that theophylline may be useful for the treatment of erythrocytosis after renal transplantation (97).

Besides hypoxia, Epo production is most reliably stimulated by the administration of cobalt. It has long been appreciated that cobalt increases the red cell mass in both man (98) and experimental animals (99). Experiments with intact animals (99), perfused kidneys (100) and Hep3B cells (101). have shown that cobalt stimulates erythropoiesis by increasing the production of Epo, thus suggesting that cobalt may be useful in studying the oxygen sensing mechanism.

Figure 1.8: Schematic model for the role of second messengers and hypoxia in the regulation of kidney production of erythropoietin.

Epo = erythropoietin; R = receptor; Gs = G stimulatory; Ac = adenylate cyclase; ATP = adenosine triphosphate; cAMP = 3', 5' -adenosine cyclic monophosphate



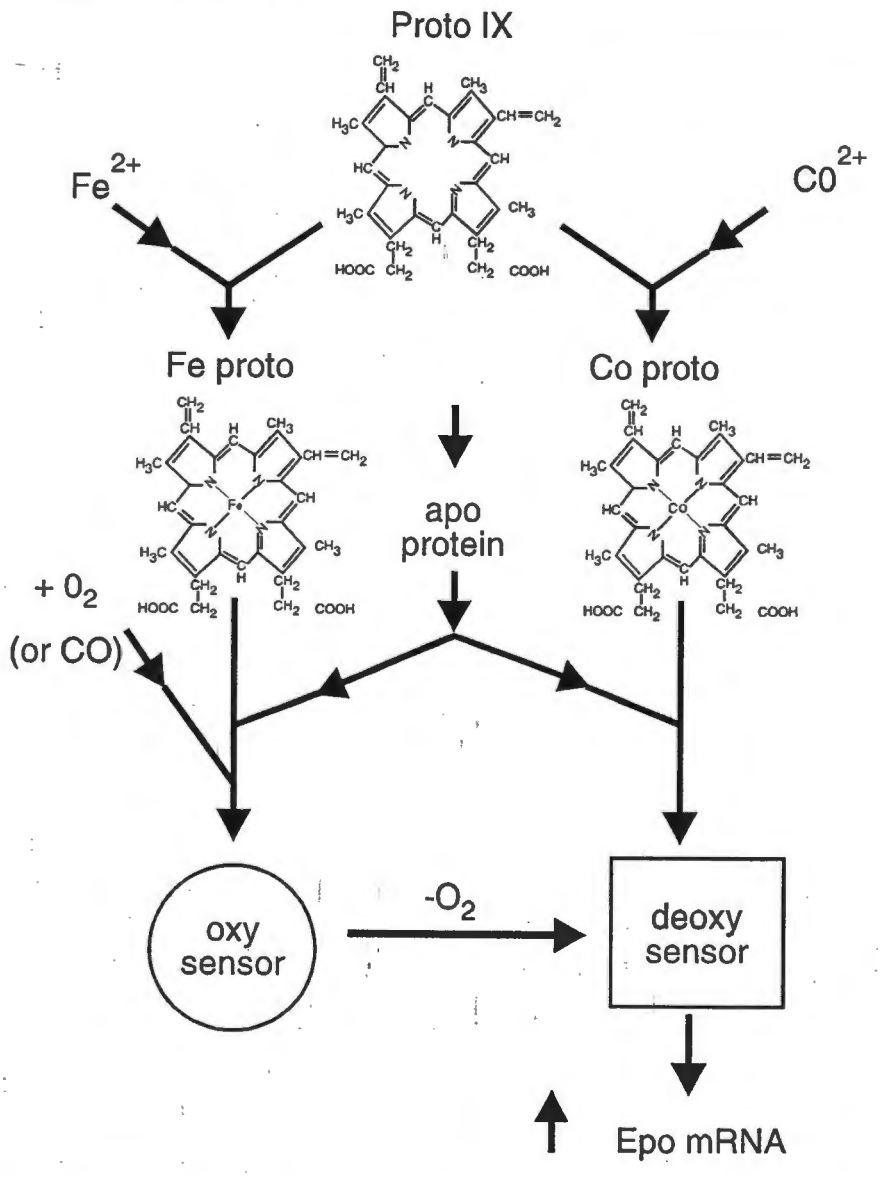
(Adapted from Fisher J & Nakashima J. Erythropoietin. Contributions to Nephrology 1994).

The initial suggestion (102) that cobalt acted through inhibition of cellular oxidative phosphorylation is not tenable since more potent inhibitors of cell respiration, such as cyanide, have no effect on Epo production (103). Many proteins that participate in reactions with molecular oxygen do so via a haem moiety.for example haemoglobin. When the ferrous iron atom in the centre of the porphyrin ring of haem binds oxygen, the haemoglobin molecule changes its conformation from deoxy or tense (T) state to the oxy or relaxed (R) state (104,105). Furthermore, it has been shown that cobalt, can be substituted for ferrous iron in the centre of the prophyrin ring of haem. However, cobalt haemoglobin binds oxygen with low affinity (106), hence, the resulting metal-substituted porphyrins are able to bind carbon monoxide.

Based on these facts Goldberg et al. (101) have proposed that the oxygen sensor for the regulation of erythropoietin production is a haem protein that is dependent on the oxygen tension to which Epo producing cells are exposed (Figure 1.9). When the oxygen tension is sufficiently low, this haem protein is in the deoxy conformation and it triggers increased expression of the erythropoietin gene. Conversely, when the oxygen tension is sufficiently high, the haem protein is in its inactive oxy conformation and does not stimulate Epo production.

When cobalt is introduced into this environment it can substitute for ferrous iron in the porphyrin ring. The resulting substituted haem protein is locked in the deoxy conformation and, like the native deoxygenated iron haem protein, acts to increase Epo expression. Although this postulate is a very plausible explanation for the oxygen sensing mechanism, this haem protein has not yet been identified.

Figure 1.9: Proposed model for the oxygen sensor in erythropoietin-producing cells. (Adapted from ref 101).



Recently Ohigashi *et al* (107) showed that the nitric oxide (NO) and guanosine 3',5' cyclic monophosphate (cGMP) system played an important role in oxygen sensing and hypoxic regulation of Epo production. NO is now known to mediate several biological processes (108,109) not only in blood vessels but in several other tissues, by activating soluble guanylate cyclase (SGC) to generate cGMP. Both SGC and nitric oxide synthase (NOS) are haem proteins (110). Yoshioka and Fisher (111) have attempted to clarify the role of NO/cGMP system in hypoxic regulation of Epo production using the isolated perfused rat kidney model. When arterial PO_2 was reduced from 100mm Hg (normoxaemic) to 30mm Hg (hypoxaemic) in the perfusate of the system, perfusate levels of Epo were significantly increased. This hypoxia induced stimulation of Epo production was significantly decreased by a specific inhibitor of nitric oxide synthase. Analysis of gene expression by quantitative reverse transcription-polymerase chain reaction also revealed that hypoxaemic perfusion produced significant increases in Epo mRNA levels in the kidney, which was blocked by a specific inhibitor of NOS. Thus it is possible that hypoxia could induce transcription of the Epo gene via modulation of transcription factors.

The mouse Epo gene has a high similarity to the corresponding human gene (112), and its has been used as a model to elucidate regulation of Epo gene transcription. Beru et al (113) identified two nuclear factors from the kidneys of mice which bound to the promoter of the Epo gene, specifically to the -61 to -45 region. One of these proteins has a MW 47 kDa, whereas the other is a ribonucleoprotein. When mice were made anaemic with a cobalt stimulus or subjected to hypoxia, binding of the ribonucleoprotein to the transcription site is greatly reduced resulting in induction of the Epo gene. The ribonucleoprotein may therefore be a negative transcriptional regulator of the Epo gene.

Studies to identify those DNA sequences within and surrounding the Epo gene that are important in Epo gene expression and Epo mRNA stability are being actively pursued. The human hepatoma cell lines Hep3B and HepG2 have provided an appropriate model for studies of this system (114). As with physiological induction of Epo production in the whole organism, Epo production by HepG2 and Hep3B in tissue culture is induced by hypoxia and cobalt but not by other stresses such as cyanide exposure or heat shock (115). Transfection of these hepatoma cells, with enhancer/promoter constructs, has defined an oxygen regulated enhancer commencing approximately 120 bp 3' to the poly A addition site of the human and mouse Epo genes (116-120). The active enhancer sequence lies in a region of at least 150 bp of striking homology between human and murine Epo gene loci. Deletional analysis of the murine enhancer showed that approximately 60 - 70 bp's were required for enhancer action when the sequence was placed 1.4 kb 5' to an α globin reporter gene (118). Hypoxia inducible operation of this sequence was further demonstrated in a wide variety of mammalian cell types which are not derived from liver or kidney and which do not produce Epo (121). Further work needs to be carried out to precisely define the sequences of the enhancer involved in hypoxic regulation of the Epo gene.

1.4.5 The Erythropoietin Receptor:

With the availability of biologically active, highly purified, radiolabelled recombinant human erythropoietin, studies have been undertaken to better characterise the erythropoietin receptor. Because of the difficulties in purifying primary BFU-E and CFU-E, many of these studies have been performed on transformed murine erythroid cell lines, some of which are erythropoietin-dependent (122-126) and some of which are not (127,128).

Sawyer et al (129) identified two polypeptides of MW 85 and 100 kDa but V8 protease digestion suggested that the smaller polypeptide is possibly a degradation product of the larger one.

In a major advance, D'Andrea et al (130) cloned the murine Epo receptor. Two independent cDNA clones encoding the Epo receptor were isolated from a pXM expression library made from uninduced murine (MEL) cells. The clones were identified by screening the transfectants for binding and for uptake of radiolabelled Epo, on the assumption that Epo could be internalised by receptor mediated endocytosis. From the cDNA sequence it may be inferred that the murine Epo receptor is a 507 amino acid polypetides with a single membrane-spanning domain.

The human Epo receptor was isolated from erythroleukemia cells and foetal liver, and its coding sequence determined (131). The human gene has been localized to chromosome 19p by *in situ* hybridization (132). The predicted sequence of 508 amino acids of the receptor protein shows 82% homology to the murine Epo receptor (131,132). Heterologous expression of the human cDNA in COS cells yielded a protein of MW 66 kDa (131). The reason for the difference in molecular mass of the cDNA-derived Epo receptor and that described by Sawyer et al is not yet understood.

Seeking homology between the Epo receptor and other receptors D'Andrea *et al* and Bazan (133,134) used sequence and structure pattern matching techniques to extract common features from the different receptor sequences. Homologous extracellularfactor-binding domains were identified in ten receptors - those for Epo, IL-1, IL-4, IL-6, IL-7, GRH, PRL, granulocyte macrophage colony-stimulating factor (GM-CSF), G-CSF and the β chain of the IL-2 receptor. This haemopoietin receptor superfamily is characterised by an N-terminal set of conserved cysteine residues except

in the case of IL-7 receptor (135) and a C-terminal collection of spaced aromatic residues, termed "WSXWS" box in one letter code notation, with X representing a non-conserved residue (135). However, many questions regarding the structure and function of the Epo receptor still remain to the answered, particularly how is the signal to proliferate and differentiate transduced when Epo binds to its receptor? Additional studies currently in progress are attempting to answer the question.

1.4.6 Erythropoietin and Anaemia of Chronic Disorders:

Several studies have investigated serum Epo in inflammatory disease. The first studies were performed using bioassays. These generally indicated that serum Epo levels were lower than expected (136-138). The problems concerning bioassays are well known: the sensitivity is low, for assay methods using polycythaemic mice, and there are problems with the specificity of the *in vitro* system. Thus no conclusive evidence could be put forward regarding the role of Epo in ACD. However, further studies have now been performed using accurate immunoassays. Most of these studies have employed radioimmunoassays with polyclonal antisera, and most recently, recombinant standards and ^{125}I - recombinant human Epo. Using such assays, normal serum levels of Epo were shown to be in the vicinity of 4-48 mU/ml, with different assays yielding slightly different, but overlapping, normal ranges (139-143).

The availability of these accurate immunoassays, and the potential to intervene with recombinant human Epo, enabled investigators to survey anaemia of diverse aetiologies to determine if the Epo response was appropriate. One question raised by this approach was that, given the wide range of 'normal' Epo responses, what are appropriate control populations with which to compare the adequacy of the Epo response ?

Erslev *et al* (144) and Birgegard *et al* (145) found that Epo responses in anaemic patients with rheumatoid arthritis and other inflammatory arthritides appeared to be similar to the responses in other types of anaemia without active inflammation. On the other hand a study by Baer *et al* (146) suggested that Epo responses in patients with rheumatoid arthritis and anaemia, the Epo response is blunted compared to a control population of anaemia of other aetiologies. Hochberg *et al* (147) also reported that patients with rheumatoid arthritis had an impaired serum-immunoreactive Epo response to anaemia compared to a control group with iron deficiency anaemia. Similarly, there have been reports of blunted Epo responses for a given degree of anaemia in patients with cancer (148), HIV infections (149), multiple myeloma (150) and ulcerative colitis (151) The cause for the blunted Epo response has not been fully elucidated. Recent experimental observations showed that certain cytokines namely recombinant TNFα and IL-1 are capable of inhibiting Epo production by HepG2 and Hep3B cells *in vitro* (152,153). IL-1 is also known to induce fever, leukocytosis, acute phase protein synthesis, and hypoferraemia, which are all typical features of chronic infectious and inflammatory diseases (154,155). Recombinant IL-1β markedly suppressed colony formation of erythroid progenitor cells (156) and seems to be identical with leukocyte endogenous mediator or leukocyte endogenous mediator described by Lee (32) more than a decade ago, as probably the 'final common pathway' that connects the various diseases associated with ACD.

1.4.7 Response of Anaemia of Chronic Disorders to Erythropoietin:

The relative deficiency in the Epo response to anaemia in ACD led to consideration of the use of Epo as treatment for this anaemia. The possibility that exogenously administered Epo might correct anaemia in rheumatoid arthritis could not be examined until supplies of the hormone became available through recombinant DNA technology (73). Winearls and his co-workers carried out the first clinical trials to establish the efficacy of Recombinant Human Erythropoietin (rHuEpo) in the reversal of anaemia due to chronic renal failure (157).

In the case of ACD, because of the blunted Epo response when compared with iron deficiency anaemia it was felt that administration of exogenous Epo would have no effect on the haematocrit (158). Nevertheless, studies on two patients suggested that anaemia in rheumatoid arthritis can be corrected using rHuEPO (159). A multicentre study confirmed that patients with this disease showed excellent haematologic responses to rHuEpo without toxicity (160).

Further studies followed with the administration of rHuEpo to patients with multiple myeloma (161) AIDS (162) and Crohn's disease (163). In the anaemia of AIDS, it was observed that patients with Epo levels of 500 U per litre or less responded to moderate doses of rHuEpo whereas those with baseline levels of more than 500 U per litre did not. Serum gamma interferon is increased in patients with AIDS and an inverse relationship has been shown with the haemoglobin level (164). Gamma interferon inhibits colony forming units erythroid (CFU-E) formation (165). The amount of rHuEpo required to overcome the inhibitory effect depends on the amount of gamma interferon present (166). This suggested that cases of ACD not responsive to rHuEpo may have extremely high levels of cytokines inhibitors of erythropoiesis.

1.5 CONCLUSIONS:

From this literature review, it can be seen that the incidence of tuberculosis in South Africa, like the rest of the world, is increasing rapidly especially amongst the coloured population of the Western Cape. A major contributor to the morbidity of the disease is anaemia, the pathogenesis of which has not yet been elucidated. This anaemia is classified as anaemia of chronic disorders. Cytokines, like tumour necrosis factor alpha and interleukin-1, have been shown to play central role in both iron metabolism and erythropoiesis, in diseases like rheumatoid arthritis, cancer and acquired immune deficiency syndrome. Furthermore, in such patients there appears to be a blunted erythropoietin response to the anaemia when compared to a control group with iron

deficiency anaemia. The cause for this has not been fully elucidated. An important aspect of the regulation of erythropoietin gene transcription lies in the enhancer element of the gene which appears to be crucial for oxygen sensing and erythropoietin gene expression.

CHAPTER 2

ERYTHROPOIETIN AND IRON MEASUREMENTS IN PATIENTS WITH ACTIVE PULMONARY TUBERCULOSIS.

2.1 Introduction

Tuberculosis in South Africa especially in the Western Cape, is increasing at alarming proportions (4). It is a major cause of death (3), but for the great majority of Mycobacterium tuberculosis infected individuals the disease is associated with great morbidity. A major contributory factor to the morbidity is anaemia. The prevalence and severity of the anaemia associated with pulmonary tuberculosis (PTB) have been described (21,22) but no work as yet been carried out investigating its pathogenesis.

The anaemia of tuberculosis is classified as anaemia of chronic disorders. In other disease states associated with this anaemia, namely, rheumatoid arthritis, Crohn's disease and cancer, a number of factors have been identified as contributing to the anaemia: a) decreased red cell survival, b) disturbances in iron metabolism and c) blunted erythropoietin response to the anaemia. With the arrival of recombinant human erythropoietin the relative importance of the last mechanism is becoming increasingly evident.

To explore the possibility that in PTB similar Epo response to anaemia occurs, the correlation between erythropoiesis, iron status and disease activity was prospectively studied.

2.2 Materials and Methods

2.2.1 Patient Selection:

Forty individuals were divided into four equal groups. The first consisted of newly diagnosed non-pregnant anaemic patients with PTB and no dissemination or other associated systemic illnesses. The second, was a matching group of patients with normal haemoglobin levels. The third, were otherwise healthy people having absolute iron deficiency: menorrhagia in three, gastrointestinal tract bleeding from benign lesions in four, and three were blood donors. The haemoglobin levels corresponded to those in Group I. Ten healthy volunteers made up group IV. All four groups were matched for age and gender.

PTB was diagnosed according to the criteria of Escreet & Cowie (167) (Appendix 1). Treatment involved a standard daily drug regimen of isonicotinic acid hydrazide (INH) 300mg, rifampicin 450mg or 600mg, streptomycin 1G intramuscularly and pyrizinamide 1.5G. Compliance with medication was ensured by supervised administration of medicines by nursing staff at Brooklyn Chest Hospital and the Chapel Street Clinic, Cape Town.

2.2.2 Laboratory Studies:

All patients and healthy individuals had the following routine investigations:
Full blood count including differential count (Technicon H1™ automated haematology analyser (168); vitamin B12, serum and red cell folate (Radioimmunoassay, Amersham International); biochemical profile (SMAC 11 AutoAnalyser, Technicon, Tarrytown, USA); C-reactive protein (CRP) quantitatively by immunodiffusion (169); serum iron,

total iron binding capacity and red cell and serum ferritin were determined according to standard methods (170,171). These investigations were carried out by the Haematology and Chemical Pathology laboratories of Groote Schuur Hospital, Cape Town.

Serum erythropoietin levels were determined by an established radioimmunoassay (Incstar EPO-TRAC RIA, Minnesota, USA). This was carried out at the Pharmacology Department of the University of Cape Town, Medical School.

All measurements, in patients, were carried out before commencing treatment and in the case of the anaemic PTB patients repeated every four weeks for a period of three months. Full haematological details including serum biochemistry of patients in each group appear in Appendix 2.

2.2.3 Ethical Considerations:

1. Informed consent:

Written or oral (in the case of patients that could not write) informed consent was secured from each patient before inclusion into the study.

Patients were fully informed in non-technical language of the nature of the drugs, the adverse events, objective and design of the study including the various blood tests, and their freedom to withdraw at any stage for any reason.

2. Ethics Committee Approval:

The protocol, subject information and consent form were submitted to Ethical Review Committee of the University of Cape Town Medical School. The study was only initiated after approval by the Committee.

3. Confidentiality:

Confidentiality of all subjects' identity was maintained by assigning a number to identify the subject in each Group. Only the subject's number was used to identify the subject for the duration of the study. The subject number will be used in the reporting of the data for the purpose of this thesis.

2.2.4 Statistical Analysis:

Data are reported as mean±SEM. The Mann-Whitney U-Test was used for the comparison of Hb, Epo and CRP levels between the various groups of patients and healthy volunteers. The analysis of variance (ANOVA) was used for the comparison of Hb, CRP, serum iron and ferritin before and at the various stages of anti-tuberculous therapy in the anaemic PTB group. Wherever the variations were not homogeneous log transformations were performed. Data were analysed using the STATPAK statistical package. Regression analysis was done using haemoglobin as independent variable and erythropoietin as dependent variable. (Statistical methods by Snedecor GW & Cochran WG, Iowa State University Press, 1980).

2.3 RESULTS

Patients in Group I had a mild anaemia with a mean haemoglobin concentration of 94±4 g/L and MCV of 82±3.6. Despite a mean serum ferritin of 638±137 ug/L the mean serum iron was only 5.6±0.4 umol/L and the TIBC 44±1.1% (Table 2.1). Patients with iron deficiency anaemia (Group II) had the same mean haemoglobin concentration, however the mean serum ferritin was only 9±0.9 ug/L with a mean serum iron of 4.1±0.4 umol/L and mean transferrin saturation of 74±1.9% (Table 2.1).

It must be noted that the mean serum vitamin B12, serum folate and red cell folate were well within the normal ranges (see Appendix 2) in all four groups (Table 2.1). For the same degree of anaemia, there was a significantly lower serum Epo level in patients in Group I than in Group III ($p<0.01$, Figure 2.1)

Table 2.1: Relevant personal and haematological values (mean ± SEM) of patients and healthy volunteers.

	GROUP I	GROUP II	GROUP III	GROUP IV
Age (yrs)	34±3	38±3	30±3	31±3
Gender	7M:3F	7M:3F	5M:5F	5M:5F
Hb (g/L)	94±4	134±14	94±5	140±6
MCV (fl)	82±3.6	91±2.2	75±2.3	90±1.6
MCH(pg)	26±1.3	31±0.8	23±1.4	31±0.7
CRP (mg%)	7.8±0.6	3.3±0.3	-	-
Iron (μmol/L	5.6±0.4	14±1.5	4.1±0.4	15±0.8
TIBC	44±1.1	51±0.6	74±1.9	57±1.0
Saturation (%)	12±0.4	27±2.5	6.1±0.6	26±1.5
Serum Ferritin (μg/L)	638±137	202±42	9±0.9	66±8
Red Cell Ferritin (fg/cell)	0.02±0.002	0.02±0.001	0.01±0.002	0.02±0.001
Vit B12 (pg/ml)	640±118	740±101	437±20	512±33
serum Folate (ng/ml)	3.5±0.7	3.3±0.2	5.2±0.4	5.3±0.3
Red Cell Folate (ng/ml)	469±42	384±48	264±44	483±34
Serum Epo (mU/ml)	54±11	21±3	142±41	23±0.8

Group I : Anaemic PTB patients, Group II: Non-Anaemic PTB patients, Group III: Iron deficiency anaemia patients, Group IV: Healthy Volunteers.

Prior to the initiation of therapy, patients with PTB but not anaemic (Group II) had a mean CRP level of 3.3±0.3 mg%. This was significantly lower ($p < 0.002$) than the mean CRP level of 7.8±0.6 mg% in the anaemic PTB patients (Group I) (Figure 2.2).

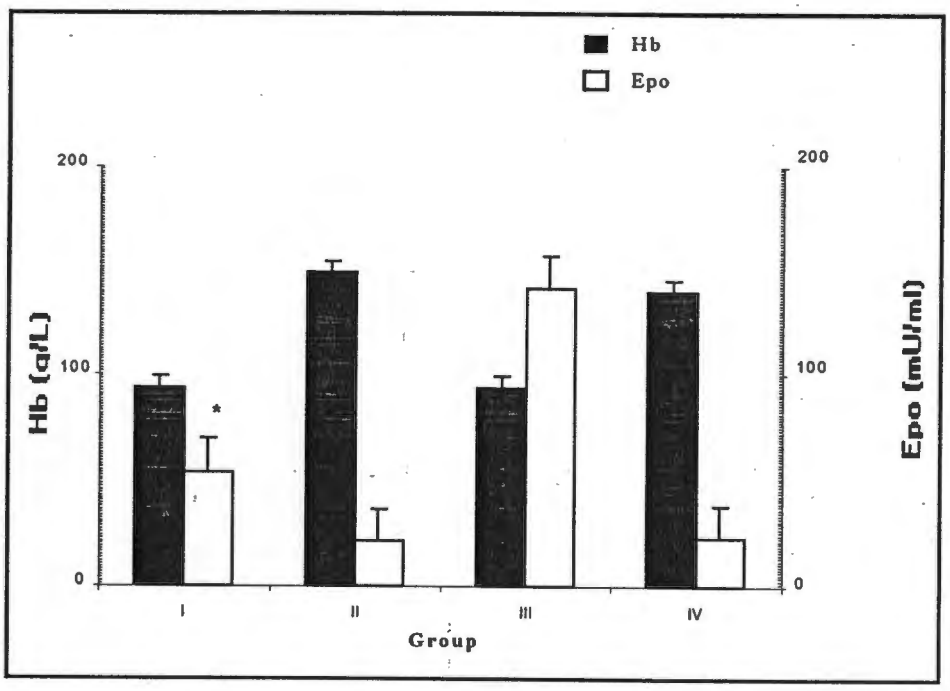


Figure 2.1: Comparison of Hb and Epo serum levels (mean ±SEM) in patients and healthy volunteers. For the same degree of anaemia, patients in group I had a significantly blunted Epo response compared to patients in group III (*p<0.01).

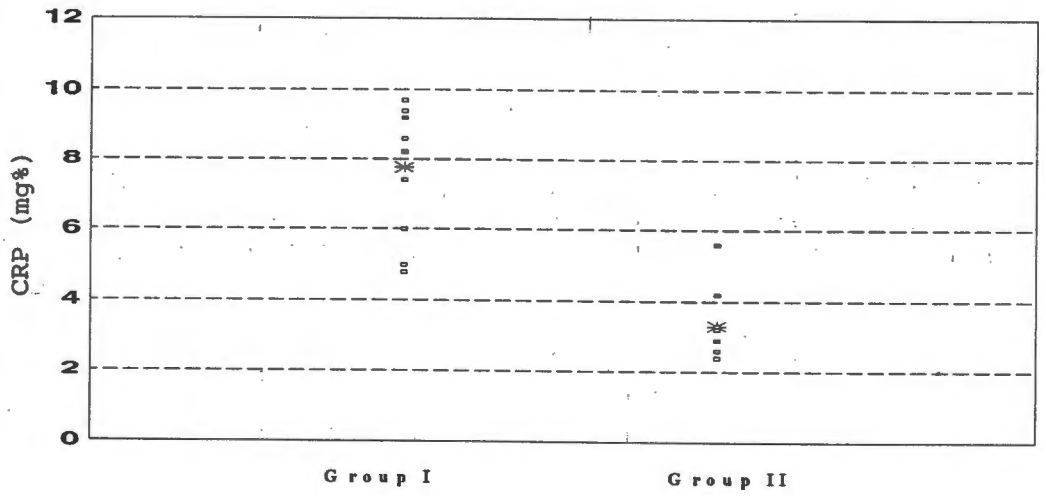


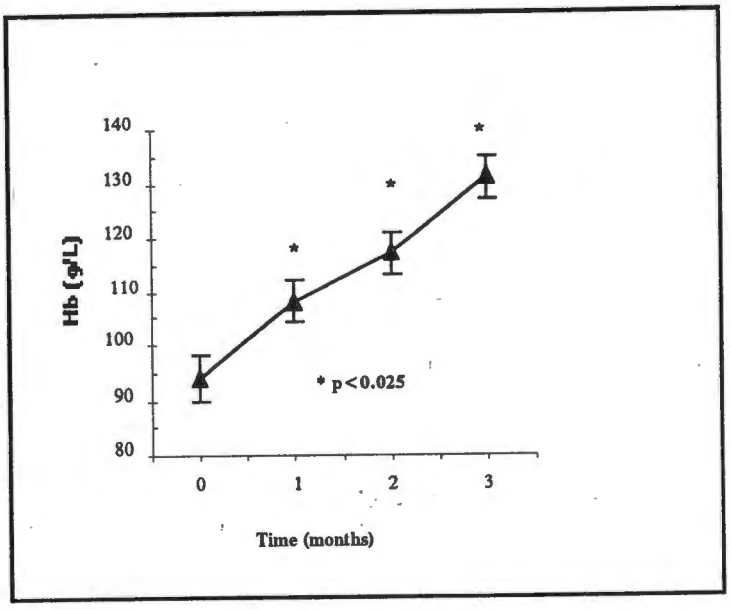
Figure 2.2. Comparison of CRP between anaemic (Group I) and non-anaemic (Group II) PTB patients before therapy .* Indicates the mean value as shown in Table 2.1. There was a significantly higher CRP level in the Group I patients compared to the Group II (p<0.002).

In the Group I patients, effective anti-tuberculous therapy, resulted in a significant rise ($p < 0.025$) in mean haemoglobin concentration to 131±4 g/L at 3 months. Furthermore, anti-microbial therapy resulted in a significant fall ($p < 0.001$) in the CRP level to 2.2±0.6 mg% at 3 months). Concurrently there was a significant rise in serum iron ($p < 0.0001$) but although the fall in serum ferritin did not reach significance levels there was downward trend. With the rise in haemoglobin there was a significant fall in Epo serum levels ($p < 0.025$). All these changes are illustrated in Fig 2.3.(Table 2.2).

Table 2.2: Effect of anti-tuberculous therapy on Hb, Epo, CRP and iron parameters of the anaemic PTB patients (Group I). Data shown as mean ± SEM.

	TIME (Months)			
	0	**1**	**2**	**3**
Hb (g/L)	94±4	108±4	117±4	131±4
Epo (mU/ml)	54±11	29±2	22±1.7	20±2
CRP (mg %)	7.8±0.6	5.5±0.8	3.9±0.7	2.2±0.6
serum Iron (umol/L)	5.6±0.4	10.1±0.7	12.4±1.3	16.1±1.7
TIBC	44±0.4	49±0.3	50±0.3	51±0.3
Saturation (%)	12±0.4	20±0.5	25±0.4	33±0.3
serum Ferritin (ug/L)	638±137	594±138	458±126	423±131

Regression analyses of the relationship between Epo and Hb in the groups I and III patients are shown in (Figure 2.4 a,b). There was significant correlation between Hb and Epo in the two groups. In the Group I patients the correlation coefficient r was 0.60 with a significance level $p < 0.03$. In the Group III patients r was 0.65 with $p < 0.$ 0.02. However the slopes and intercepts of the two lines were different. In the Group I patients the slope was -1.65 with an intercept at 210.3 (mU/ml) compared to the Group III patients with a slope of -5.92 and intercept at 700.8 (mU/ml). Thus it appears that the Epo response to anaemia is blunted in the Group I patients, however the normal physiological response to anaemia is still operational.


Hb (g/L)
140
130
120
110
100
90
80
* p<0.025
0
1
2
3
Time (months)

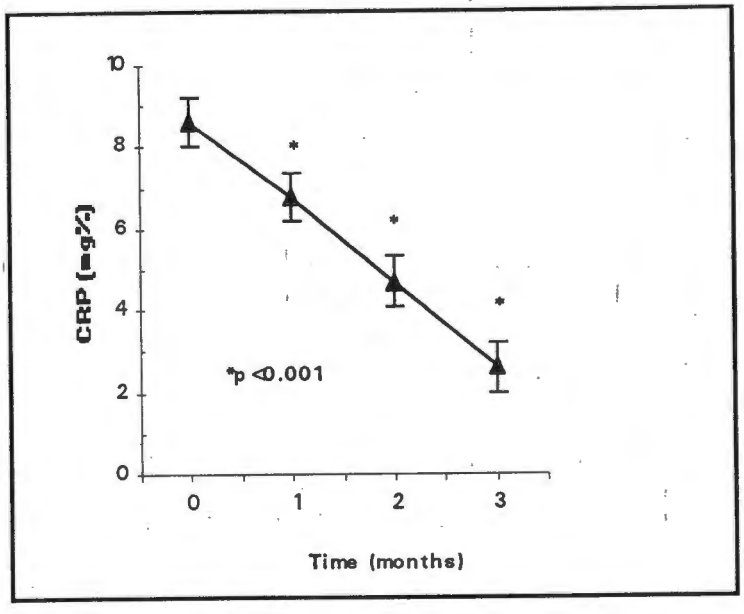
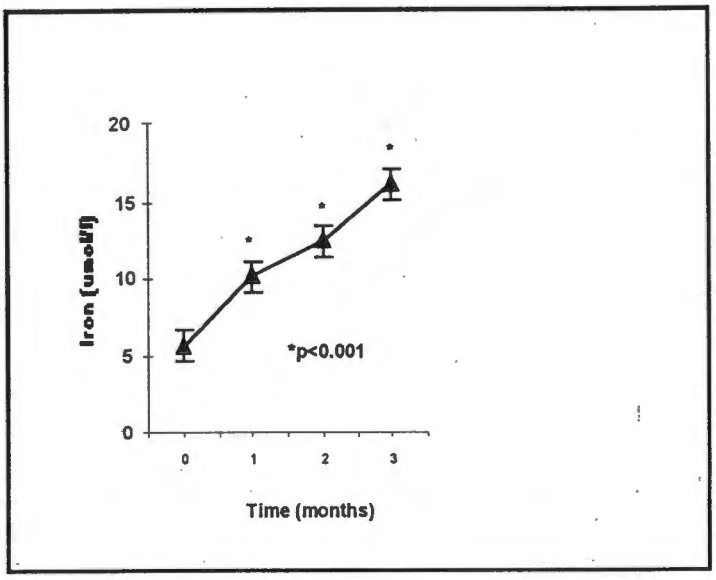

CRP (mg%)
10
8
6
4
2
0
*p <0.001
0
1
2
3
Time (months)

Figure 2.3a


20
15
10
5
0
Iron (umol/l)
*p<0.001
0
1
2
3
Time (months)

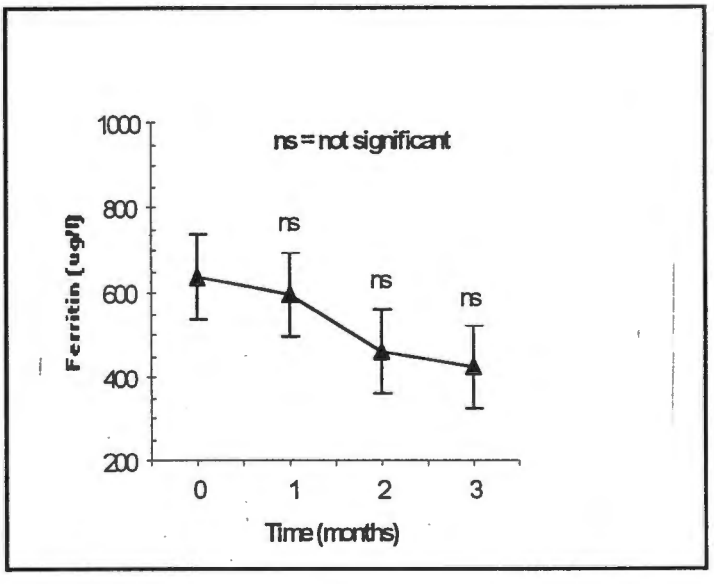

1000
800
600
400
200
Ferritin (ug/l)
ns = not significant
ns
ns
ns
0
1
2
3
Time (months)

Figure 2.3b

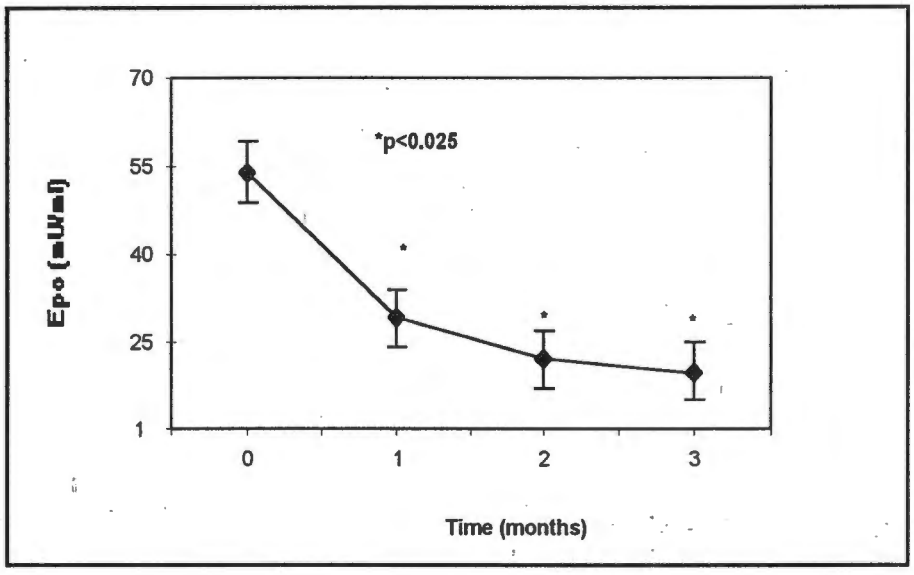


Figure 2.3c

Figure 2.3abc: Effect of anti-tuberculous therapy on Hb, CRP, serum iron, ferritin and Epo in anaemic PTB patients. Data were obtained as described in Materials and Methods. With therapy there was a significant fall in the CRP levels and this resulted in significant rise in Hb and serum iron. With the rise in Hb there was a significant fall in the Epo serum level. The significance level comparing various stages of treatment to base line is shown in each graph.

Group I

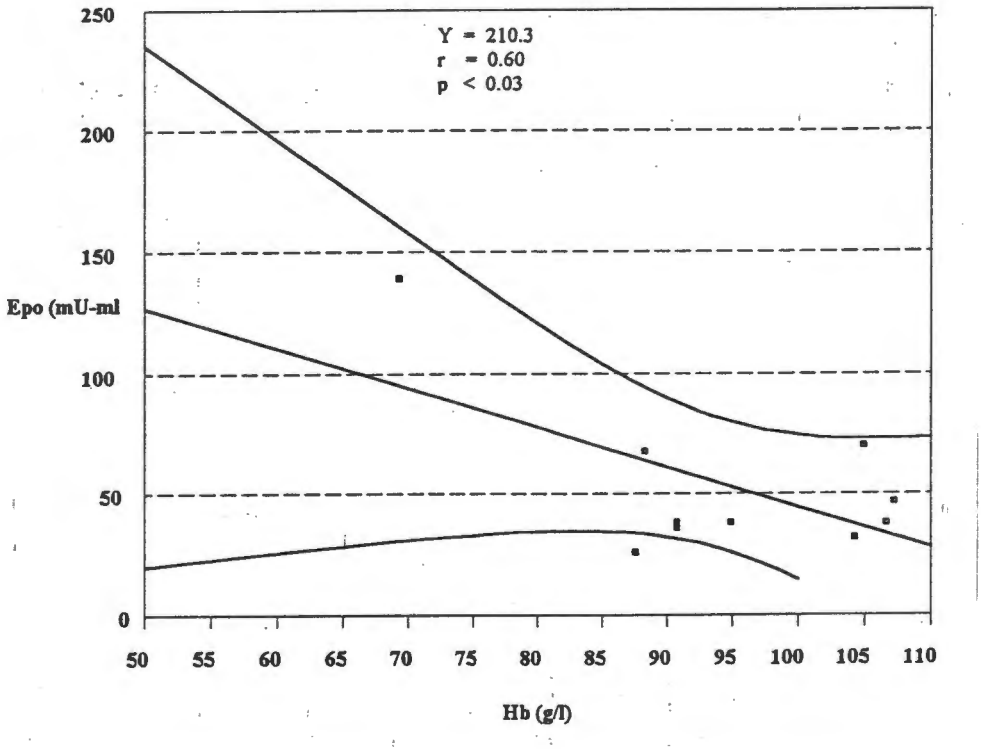


Figure 2.4a. Regression analysis of anaemic PTB patients (Group I). Epo and Hb levels were determined as described in Materials and Methods. The slope was -1.65 the intercept was 210.3 and the correlation coeffeicient r=0.60 with a significant level of p<0.03. The curved lines represent 95% confidence intervals.

Group III

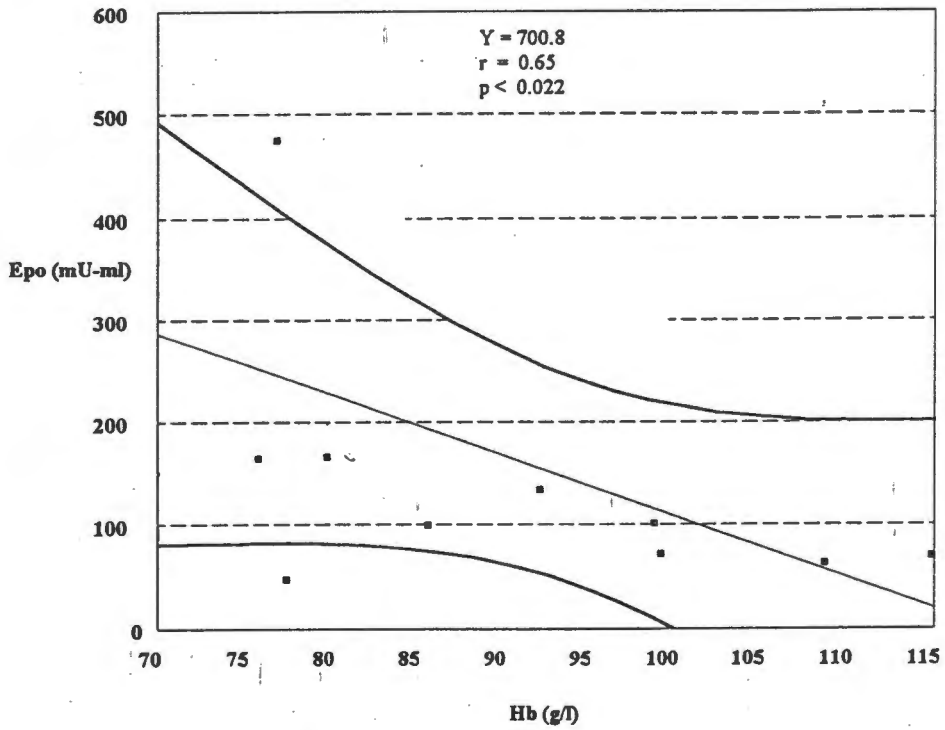


Figure 2.4b Regression analysis of anaemic Iron deficiency patients (Group III). Epo and Hb levels were determined as described in Materials and Methods. The slope was -5.92, the intercept was 700.8 and the correlation coefficient r=0.65 with a significance level of p<0.022.
The curved lines represent 95% confidence intervals.

2.4 DISCUSSION

The diagnostic feature of anaemia of chronic disorders (ACD) is hypoferraemia with low serum transferrin or low serum total iron binding capacity in the setting of adequate iron stores (23). The mild anaemia of the patients with active PTB typifies this anaemia in that the serum iron was reduced with adequate body iron stores as shown by the serum and red cell ferritin with the former being a less reliable marker since it behaves as an acute phase reactant (171).

The development of anaemia during chronic disease is not well understood. Two possible explanations can account for the observations: i) interference in iron metabolism and ii) decreased levels of Epo.

Cartwright (40) postulated that the anaemia of chronic disease may be due to a block of release of iron from the RES. Although animal models of ACD supported this theory (41), no evidence of a blocked RES iron release could be demonstrated in patients with rheumatoid arthritis (42) and pulmonary tuberculosis (21). In the latter study, Baynes *et al* argued that the anaemia of PTB could not be due to iron deficient erythropoiesis as the percentage saturation of iron between anaemic and non-anaemic individuals were the same (that is 31%). It must be pointed out that all patients had been on anti-tuberculous therapy for at least one month prior to any measurements being made.

In this study, the mean percentage saturation of iron in the anaemic PTB patients (Group I) was only 12 % before commencement of therapy. Within one month of anti-tuberculous therapy, there was a rapid rise in serum iron with a percentage saturation of 20 % (Table 2.2). This in agreement with the observations of Morris *et al* (22).

Thus it appears that prior to commencement of anti-tuberculous therapy, iron deficient erythropoiesis may play a role in the pathogenesis of anaemia of PTB.

The impaired mobilisation of iron in patients with ACD may be the result of cytokines released during the inflammatory/infective process. Moldawer *et al* (61) showed that injection of recombinant TNFα in rodents resulted in hypoferraemic anaemia associated with impaired RES iron release and incorporation into erythrocytes. Denz *et al* (59) showed that the degree of activation of macrophages - as measured by the serum neopterin concentration - in patients with malignancy resulted in the development of anaemia by a shift of iron toward storage sites suggesting a role of immune activation in altered iron metabolism. Rogers *et al* (60) reported that interleukin 1 (IL-1) increased translation of ferritin mRNA and have suggested that this additional ferritin might act as a trap for iron that might otherwise be available for erythropoiesis.

In this study the significantly lower CRP level in the non-anaemic PTB group compared to those with active PTB and anaemia, argues for the degree of inflammation being causally related to the anaemia. With anti-tuberculous therapy there was a significant rise in serum iron and downward trend of serum ferritin. These findings are compatible with the above studies in that anti-tuberculous therapy may have resulted in a decrease in circulating inflammatory cytokines and mobilisation of iron from storage sites and improvement in anaemia.

Typically there is an inverse relationship between serum Epo levels and haemoglobin: as the haemoglobin decreases, the Epo level rises (144). The physiological response to anaemia is clearly shown in this study in that there was a significant negative correlation between serum Epo levels and haemoglobin in both iron deficiency and anaemic PTB patients.

However the slopes and intercepts of the regression analyses of the two parameters were clearly different between the two groups. In the anaemic PTB group the slope and intercept were less than half that of the iron deficiency group. It must be remembered that both groups had similar degrees of anaemia, thus the physiological response in the anaemic PTB group although operational is ***attenuated.*** These findings are consistent with those of Baer *et al* (146) who showed that in anaemic rheumatoid arthritis patients there was an inverse relationship between haemoglobin and Epo levels; however, for any given anaemic individual with rheumatoid arthritis the Epo level was lower than that found in equally anaemic individual with iron deficiency. The findings of a blunted Epo response to anaemia were also shown by Hochberg *et al* (147) and Boyd and Lappin (172) in the case of rheumatoid arthritis. Similar results have been reported in patients with cancer (148), HIV infections (149), multiple myeloma (146) and ulceratice colitis (151).

The cause for the attenuated Epo response has not been fully elucidated. Recent experimental observations showed that certain cytokines namely recombinant TNFα and IL-1 are capable of inhibiting Epo production by the hepatoma cell lines HepG2 and Hep3B *in vitro* (152,153); *in vitro* monocyte secretion of IL-1β was associated with severity of anaemia in patients with inflammatory bowel disease (151).

The relative deficiency in the Epo response to anaemia in ACD led to consideration of the use of Epo as treatment for this anaemia. A multicentre study showed that patients with rheumatoid arthritis had excellent haematologic responses to recombinant human erythropoietin (rHuEpo) without toxicity (160). Further studies followed with the administration of rHuEpo to patients with multiple myeloma (161), AIDS (162) and Crohn's disease (163). It appears that administration of pharmacological doses of Epo is able to overcome the inhibitory effects of inflammatory cytokines on erythropoiesis.

In this study as in other examples of ACD impairment of iron metabolism and erythropoiesis are each present, but several reasons exist for believing that the latter is more important: i) rHuEpo can correct ACD but cannot correct the anaemia of iron deficiency; (ii) iron ingestion, which increases the serum ferritin levels, has no effect on the anaemia of rheumatoid arthritis, but Epo normalises the haematocrit; (iii) serum transferrin receptor levels are elevated in patients with iron deficiency, but are generally not elevated in patients with ACD.

In conclusion, it appears that there are two contributory factors to the anaemia of active PTB, namely, a disturbance in iron metabolism and a blunted erythropoietin response to the anaemia. The marked decrease in the inflammatory marker CRP with anti-tuberculous therapy, and the resultant rise in haemoglobin levels and serum iron leads one to believe that inflammatory cytokines may have been responsible for the haematological changes seen with this chronic disease. The effect of cytokines on erythropoietin production will be elucidated further in Chapter 3.

CHAPTER 3

MODULATION OF ERYTHROPOIETIN PRODUCTION BY TUMOUR NECROSIS FACTOR ALPHA: *IN VITRO* STUDIES AND ITS CLINICAL IMPLICATIONS

3.1 Introduction

The production of erythropoietin is stimulated in the presence of hypoxia (91,92). In anaemic patients without renal disease an inverse relationship exists between the serum erythropoietin level and the haemoglobin concentration of the blood (144). This physiological response of Epo to anaemia occurs in patients with active PTB, however the response is attenuated when compared to patients with pure iron deficiency anaemia (173).This is in agreement with clinical studies on other examples of ACD (146-150). Hence it has been proposed that as yet unidentified factors may influence the production of erythropoietin independently of hypoxia (174).

Mycobacterial lipoarabinomannan is a potent trigger of tumour necrosis factor alpha (TNFα) release from monocyte-macrophages (175). TNFα production in the granulomata is an essential component of the immune response to mycobacterial infections (15). The peripheral blood mononuclear cells (PBMC) from these patients are known to release large quantities of TNFα *in vitro* (176). The fever, weight loss and tissue injury that characterise advanced tuberculosis may be attributable in part to TNFα (17). TNFα also exerts an inhibitory effect on haematopoiesis, both *in vitro* (177) and *in vivo* (61). Recently it has been shown that recombinant TNFα can inhibit the production of Epo by the human hepatocellular lines, HepG2 and Hep3B *in vitro* (152,153).

This study was undertaken to investigate whether TNFα produced by the PBMC of patients with active pulmonary tuberculosis is capable of inhibiting the production of erythropoietin by HepG2 cells *in vitro* and whether the inhibition, if any, can be reversed by specific anti-TNFα antibodies.

3.2 Materials and Methods

3.2.1 Patients:

Blood mononuclear cells were recovered form a total of 15 patients: Group I (n=5) anaemic PTB patients, Group II (n=5) non-anaemic PTB patients and Group III (n=5) iron deficiency anaemia patients. The patients were matched for age and gender.

3.2.2 Monocyte Cultures:

Heparinized blood samples from PTB patients and iron deficient controls were diluted (vol/vol) in calcium-free Hanks' buffered saline solution (HBSS), supplemented with 0.08% EDTA. The peripheral blood mononuclear cells (PBMC) were isolated by layering the samples over Ficoll Hypaque density gradient and centrifuged at 1000 g for 15 minutes. In order to purify the PBMC, the adherent cells were resuspended in the same medium diluted 1/9 (vol/vol) in calcium-free HBSS and centrifuged (100 g for 10 minutes) twice to remove any platelets. Thereafter, the PBMC were resuspended in culture medium made up with RPMI (Highveld Biologicals, Johannesburg, SA) buffered with HEPES to pH 7.4 and supplemented with 1% non-heat inactivated foetal calf serum (FCS) (Highveld Biologicals, Johannesburg, SA), 100U/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine. The cells were counted after staining with acridine orange and adjusted to a concentration of 10^6 cells/ml. 100µl volumes of this suspension were added to 96-well microplates (Linbro Brand, Flow Laboratories, Inc., McLean, Virginia, USA). The total volume of each well was adjusted to 200µl with complete medium. The cells were incubated with 5% CO_2 and 20%O_2 for 48 hours without stimulation. The monocyte supernatant fraction (SNF) from patients and control were removed and stored at -70^{0}C until assayed for TNFα.

3.2.3 Tumour Necrosis Factor alpha assay:

TNFα released from unstimulated PBMC was assayed by a immunoradiometric assay: SNF (25µl) from patients and iron deficient controls were incubated overnight with ^{125}1-labelled anti-TNFα monoclonal antibody in tubes coated with monoclonal antibody directed against different epitopes of TNFα (IRMA, Amersham, Buckingham, UK). After washing, the remaining radioactivity bound to the tube was counted in a gamma counter.

3.2.4 HepG2 cell cultures:

HepG2 cells that had been evaluated and standardised in culture (Department of Medical Biochemistry, Medical School, University of Cape Town, obtained through American Type Culture Collection) were plated on 24 well polystyrol dishes (Linbro Brand, Flow Laboratories, Inc., Mclean, Virginia, USA) and cultured in modified Eagles' medium (MEM) with 10% (non-heat inactivated) FCS (Highveld Biologicals, South Africa and sodium bicarbonate without antibiotics. The cells were incubated at 37^{0}C in humidified air, with 5%CO_2 until 75% confluence was obtained.

The media were then removed and the various dilutions (in duplicate) of monocyte SNF from patients and controls were added to the cells. The HepG2 cells were incubated for a further 24 hours, after which the SNFs were removed and stored at -20^0 C until analysis of the Epo concentration by radioimmunoassay (Incstar EPO-TRAC RIA, Minnesota, USA).

The same experiments were conducted with the addition of a 1:800 dilution of specific polyclonal goat 1gG-(H34) and 1:200 dilution of specific monoclonal 1gG-(101-4) TNFα antibodies (National Biological Standards Board, Hertfordshire, UK).to the monocyte SNF of the anaemic PTB group.

3.2.5 Protein Estimation:

In order to standardise for the number of HepG2 cells in each well, cellular protein estimation was done. The cells were washed with phosphate-buffered saline and lysed with SDS-NaOH (0.5% sodium dodecyl sulphate in 0.1M sodium hydroxide). Total cellular protein was determined by the automated Bio-Rad dye binding microassay system as described by Bradford (178) using bovine serum albumin as a standard. Samples were diluted 50-fold prior to assay in order to minimise any interference by SDS-NaOH. Epo levels were then expressed as mU/mg protein.

3.2.6 Statistical Analysis:

Analysis of variance (ANOVA) was used for comparison of TNFα produced by monocytes and for the levels of Epo produced by HepG2 cells in the presence of monocyte SNF from PTB patients and iron deficient controls. The results are reported as mean ± SEM.

3.3 RESULTS

Adherent PBMC from anaemic PTB patients produced significantly higher ($p < 0.001$) TNF (770±173 pg/ml) than monocytes derived from the non-anaemic PTB patients) (431±90 pg/ml). The level of this cytokine was significantly higher ($p < 0.005$) in the monocyte SNF of the Group II patients compared to the iron deficient controls (Group III) (98±17 pg/ml) (Figure 3.1 A) The production of Epo by HepG2 cells were markedly inhibited in the presence of monocyte SNF form patients of Group I compared to both Groups II and III ($p < 0.001$) (Figure 3.1B). The results shown are duplicate experiments performed on five individuals in each of the three groups.

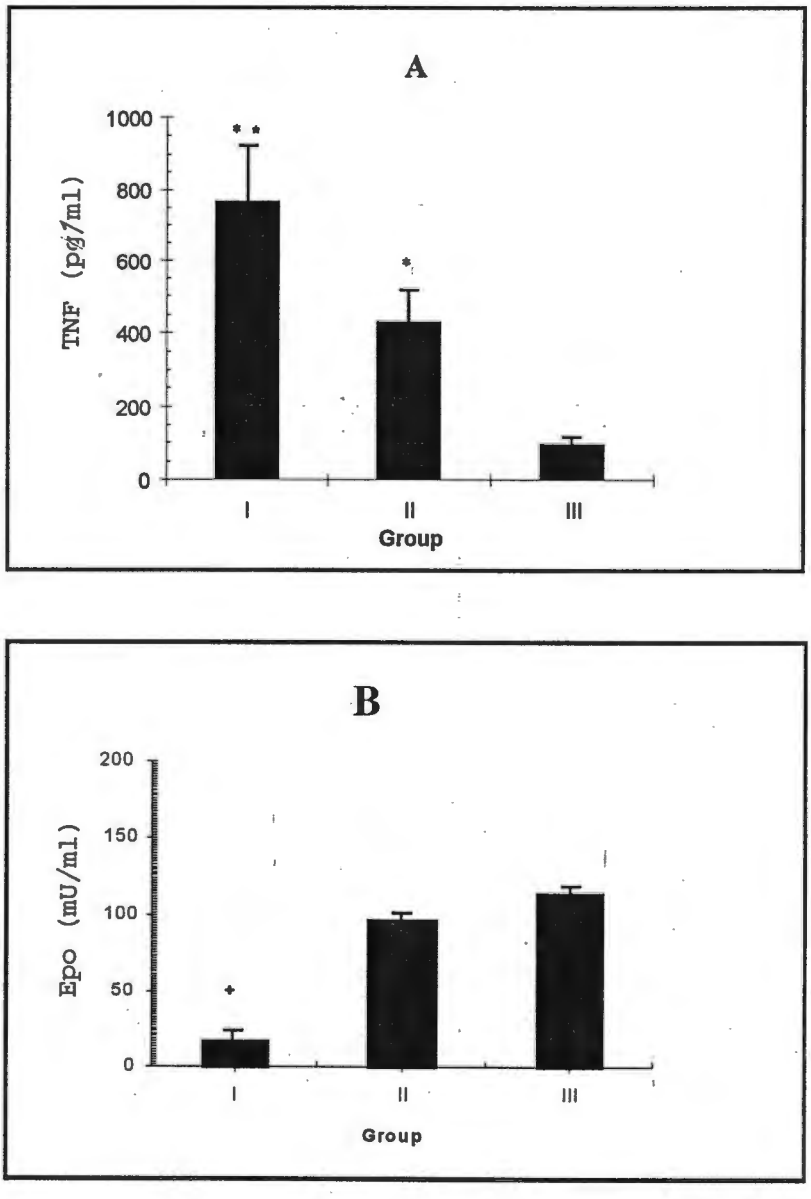


Figure 3.1: **A** Production of TNFα by PBMC obtained from untreated anaemic PTB patients (I), non-anaemic (II) and iron deficient controls (III) (**p<0.001 comparing groups I and III, *p<0.005 comparing groups II and III). **B** Also shown are the effects on Epo production by HepG2 cells in the presence of monocyte SNF from the same three groups. Data were obatained as described in Materials and Methods. The significantly higher levels of TNFα produced by the PBMC of the group I patients resulted in a marked inhibition of Epo production by HepG2 cells (+p<0.001 comparing groups I and III).

The effects of monoclonal and polyclonal anti-TNFα antibodies on Epo production by HepG2 cells in the presence of various dilutions of monocyte SNF were examined in the Group I patients (Figure 3.2). The addition of increasing concentrations of monocyte SNF resulted in a progressive reduction of Epo produced by the HepG2 cells. In the presence of optimal saturating concentrations of anti-TNFα antibodies the release of Epo was elevated. The falloff reflects the use of fixed dose antibodies, with the decline at 40% SNF attributable to increasing availability of the inhibitory cytokine.

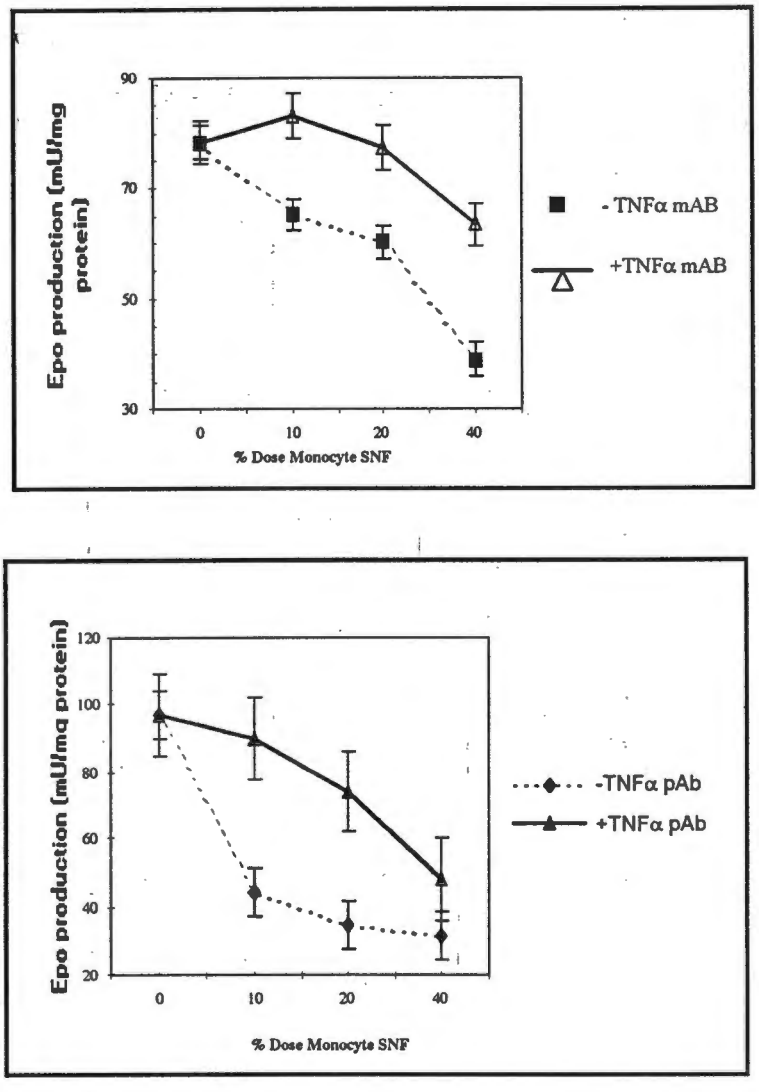


Figure 3.2 The effect of specific monoclonal antibody (mAB) or polyclonal antibody (pAb) on Epo production by HepG2 cells in the presence of increasing concentrations of monocyte SNF from anaemic PTB patients. Data mean±SEM represent measurements of Epo production after addition of monocyte SNF from 5 anaemic PTB patients at each dose in the presence and absence of specific anti TNFα antibodies. With increasing doses of SNF there was a marked fall in Epo production by HepG2 cells. This was reversed in the presence of the specific anti-TNFα antibodies. ($p < 0.001$ comparing Epo production in the presence and absence of specific anti-TNF antibodies).

3.4 DISCUSSION

The anaemia associated with PTB appears to be explicable, at least in part, by reference to inappropriate plasma Epo levels, causally linked to the inflammatory process that leads to the release of a number of inhibitory cytokines.

This study showed that *in vitro* production of TNFα by PBMC purified from anaemic PTB patients was significantly greater than in the non-anaemic PTB group and iron deficient controls. Furthermore, monocyte SNF from the anaemic PTB group inhibited the production of Epo by Hep G2 cells *in vitro*. The suppressed release of the hormone was more pronounced than in anaemic PTB patients than those with iron deficiency, in keeping with the higher levels of TNFα in the anaemic PTB patients. Although the levels of TNFα in the non-anaemic PTB group were greater than in iron-deficient controls, no observed suppressive effect on Epo production by HepG2 cells was observed under the experimental conditions studied. It appears, therefore, that a critical level of cytokine or synergistic responses may be necessary in order to block the increased requirement for Epo synthesis, thus contributing to the anaemia.

Faquin et al (152) reported that recombinant IL-1, TNFα and TGFß inhibited the production of Epo from the hepatoma cell line Hep3B. This effect appeared to occur at the level of Epo mRNA. Jelkmann and co-workers (153) using HepG2 cell line, reported similar results for IL-1 and TNFα, but noted no inhibition with TGFß. In addition they reported that IL-1ß inhibited Epo production by isolated perfused rat kidney. In a recent study, increased levels of IL-1ß was correlated with the severity of the anaemia in patients with inflammatory bowel disease (163). However in this study, the effect of the increased level of cytokine on Epo production in these patients were not investigated.

In this study, abrogation of the suppression by monocyte SNF by both poly-and monoclonal anti-TNFα antibodies implicates TNFα produced from activated PBMC, in impairing Epo release *in vitro*. An effect of this on erythropoiesis may explain the impaired response to anaemia in patients with PTB or other chronic inflammatory conditions.

In a recent study (179), we showed that addition of monocyte supernatants from patients with chronic renal failure, resulted in a sharp drop in Epo production by HepG2 cells *in vitro*. There was however no direct correlation between increased levels of TNFα, IL-1α and IL-1β and the degree of suppression. Furthermore, inhibtion of these cytokines by specific monoclonal antibodies did not block the inhibition of Epo release by HepG2 cells.

The results of this study, demonstrate that monocytes from patients with chronic renal failure produce factors that inhibit Epo production by HepG2 cells *in vitro*. This observation implies that a further mechanism, in addition to reduction in renal mass, inhibits Epo secretion and may also contribute to the pathogenesis of the anaemia of chronic renal failure. However, unlike tuberculosis patients, the factors responsible for this inhibition is not the cytokine TNFα.

TNFα has been shown to exert an inhibitory effect on haematopoesis, both in vitro (177) and in vivo (61). This effect is probably mediated by nitric oxide (NO), whose synthesis has recently been shown to be stimulated by both IFNγ and TNFα (180). When normal bone marrow or CD34+ cells were exposed to NO, apoptosis of bone marrow progenitors took place. Inducible nitric oxide synthase (NOS) mRNA was found in bone marrow after stimulation with IFNγ and TNFα. Presence of an NOS inhibitor partially reversed the effect of TNFα.

Furthermore, NO and 3', 5' cyclic monophosphate (cGMP) system has been shown to play an important role in the oxygen sensing and hypoxic regulation of Epo production (111). Using the isolated perfused rat kidney model it was shown that when arterial PO_2 was reduced from 100mm Hg (normoxaemic) to 30mmHg (hypoxaemic) in the perfusate of the system, perfusate levels of Epo were significantly increased. This hypoxia induced increase in Epo production was significantly decreased by a specific inhibitor of NOS.

Thus, in order to understand the complex mechanisms by which TNFα and NO may exert their effects on Epo production, it is essential to study the mechanism of oxygen sensing and expression of the Epo gene. This will be elucidated in Chapter 4.

CHAPTER 4

OXYGEN SENSING AND ERYTHROPOIETIN GENE EXPRESSION

4.1 Introduction

Erythropoietin (Epo) plays a central role in the feedback regulation of erythropoiesis in response to anaemia and reduced blood oxygenation (168). However, the Epo response to anaemia may be blunted in chronic disease like for example rheumatoid arthritis (146) inflammatory bowel disease (163) and pulmonary tuberculosis (173). This may be the result of the presence of TNFα which inhibits the release of Epo *in vitro* (152,153,173). In order to understand the mechanism (s) by which this modulation in Epo production occurs, it is essential to study the oxygen sensing mechanism and expression of the Epo gene.

The human hepatoma cell lines Hep3B and HepG2 have provided an appropriate model for the study of oxygen regulated Epo gene expression (114). As with the physiological induction of Epo production in the whole organism, Epo production by these cells is induced by hypoxia or cobaltous ion, but not by other stresses such as cyanide or heat shock (115). Transfection studies in these hepatoma cells have defined an oxygen regulated enhancer commencing approximately 120 bp 3' to the poly A addition site of the human and mouse Epo genes (116-120) (Figure 4.1).

The active enhancer sequence lies in a region of at least 150bp of striking homology between human and murine Epo gene at the 3' end of the gene. Deletion analysis of the murine enhancer showed that approximately 60-70bp were required for enhancer action when the sequence was placed 1.4 kb 5' to an $\alpha_1$globin reporter gene (117). Hypoxia inducible operation of this sequence was further demonstrated in a wide variety of mammalian cell types which are not derived from kidney or liver and which do not produce Epo (121).

```
                                        poly A
Mouse CTGGCAACAG CTGAAATCAC CAACCAGACT CCTGGCTTGT CTCTCTTCAT
Human TTGACAAGAA CTGAAACCAC CAATATGACT CTTGGCTTTT CTGTTTTC--

Mouse GACTGTACAC ACCACACAAC TCTCCTAGCT GTACCTCACC CCATCTGGTC
Human ---------- ---------- ---------- ---------- ----------
                                                   1 ApaI
Mouse GCAAGGCATC AGATCTGGGA AACCAGAGGT CGAGGGGGTT GGGCCCTACG
Human ---------C AGGTCCGGGA AATGAGGGGT CGAGGGGGCT GGGCCCTACG
          11        21        31        41        51
Mouse TGCTGCCTCG CATGGCCCGG CTGACCTCTT GACCCCTCTG GGCTTGAGGC
Human TGCTGTCTCA CACAGCCTGT CTGACCTCTC GACCTACCG G CCT AGGC
          61        71        81        91       101
Mouse CACAATACCT GCCCACGCTA GTCAATAAGC AGGCTCCATT CAAGGCTGTC
Human CACAAGCTCT GCCTACGCTG GTCAATAAGG TGTCTCCATT CAAGGCCTCA
          111       PvuII
Mouse TCTCAGTGGG CAGCT
Human CCGCAG
```

Figure 4.1: Comparison of the human and murine DNA sequence at the 3' end of the Epo gene. In each case the active enhancer sequence is located approximately 120 bp 3' to the poly A addition site (underlined). Sequence conservation is approximately 85% within the functionally defined enhancer, although the best alignment is achieved by making gaps as shown in the human sequence. The murine sequence in this region has been numbered from A*pa*I site to facilitate description of constructs containing part of this sequence. The dashed line in the human sequence indicates the area where no significant homology exists.

In order to further characterise the sequences of the Epo enchancer involved in oxygen sensing and gene regulation detailed studies of the mouse Epo enhancer were carried out. This involved transfection experiments of deleted, mutated and re-iterated sequences plus DNAse 1 protection analysis, to define the subsequences within the enhancer. However, since the operation of an enhancer in different cell types may result from different DNA protein interactions (182,183), the experiments were carried out in different cell types: HepG2 cells, which produce Epo and support regulated operation of the mouse Epo 3' enhancer and the Chinese hamster lung fibroblastoid line, a23, which support regulated enhancer function but do not produce Epo.

4.2 Materials and Methods

4.2.1 Cell lines and culture conditions:

HepG2 cells were grown in minimal essential medium with Earle's salts supplemented with 10% foetal calf serum, glutamine (2mM), penicillin (50 iu/ml) and streptomycin sulphate (50 ug/ml). a23 cells, were grown in the same medium with the addition of sodium pyruvate (1mM).

4.2.2 Plasmid Constructs:

After growth in E. coli, plasmid DNA was prepared by alkaline lysis (184) and purified on a caesium chloride gradient. Constructs containing deletions of the mouse Epo enhancer linked to a 2570 bp B*gl*II - P*pu*MI fragment containing the intact human α_1 globin gene with 1.4kb of 5' flanking sequence, in either orientation, into the N*ot*I site of pBluescript SKII (Stratagene Ltd., Cambridge) were made with appropriate linkers (pBSα-) (118). To assess the effects of distance of the enhancer sequence from the promoter, the 2570 bp α_1 globin fragment was exchanged for an otherwise identical fragment which was deleted to position - 124 in the α_1 globin 5' flanking sequence (pBS$\Delta\alpha_1$). The enhancer nucleotides present in these plasmids were designated by numbers related to the first nucleotide of the A*pa* I site at the 5' end of the enhancer (Figure 4.2).

```
                                                1                   25
AGATCTGGGAAACCAGAGGTGGAGGGGGTTGGGCCCTACGTGCTGCCTCGCATGGCCCGGC
Bgl II                  48              Apa I
TGACTCTTGACCCCTCTGGGCTTGAGGCCACAATACCTGCCCACGCTAGTCAATAAGCAGGCT
  96
CCATTCAAGGCTGTCTCTCAGTGGGCAGCTG
                              Pvu II
```

Figure 4.2: DNA sequence of the enhancer. Nucleotides are numbered from the first base of the A*pa* I site. Nucleotides 1-48 underwent 4 base pair mutations where the purines were substitutes for their non-complimentary pyrimidines and vice-versa.

Consecutive four base pair mutations of the Epo enhancer, in which purines were substituted for their non-complimentary pyrimidine and vice versa, were made using the pALTER based Altered Sites™ *in vitro* mutagenesis system as

directed by the manufacturers (Promega Ltd., Southhampton). For these constructs a 460 bp sequence containing the mouse Epo enhancer was ligated into pALTER. Mutagenic oligonucleotides contained the four mutated nucleotides flanked by fourteen complementary nucleotides on each side. After retrieval of the mutant plasmid and confirmation of mutation by dideoxy sequencing, a 153 bp B*gl*II - P*vu*II fragment containing the mutated Epo enhancer region, was subcloned into polylinker sites in pBluescript SKII which contained the 2570 bp α_1 globin fragment described above (pBSα-). A similar plasmid containing the unmutated 153bp B*gl*II- P*vu*II fragment from the Epo enhancer locus was made to enable direct comparison of function between wild type and mutated enhancer sequences.

Further subsequences from within the Epo enhancer were derived either by PCR amplification or by annealing synthesised oligonucleotides. Generation of concatamers and recombination of subsequences was facilitated by using oligonucleotides in which compatible overhanging ends could be generated from X*ba*I and S*pe*I restriction enzymes sites. These sequences were inserted adjacent to the SV40 promotor in pSVGH. pSVGH contained a 198 bp N*si*I-H*ind*III fragment containing the SV40 early promotor sequence linked to human growth hormone (B*am*HI-N*si*I fragment) in pGEM7 (Promega, Southampton). Plasmids containing no erythropoietin sequence (pSVGH) or the 168 bp K*pn*I-N*si*I fragment containing the SV40 enhancer instead of Epo sequence (pSVeSVGH) were used as controls.

A plasmid containing 290bp of the promotor of the mouse ferritin heavy chain subunit gene fused to the human growth hormone gene (pFGH) was used to control for transfection efficiency in experiments where the test plasmid contained the α globin reporter gene. In experiments where the test plasmid contained the growth hormone reporter gene, the α globin containing plasmid pBSα- was used to control for transfection efficiency.

4.2.3 Transient Transfection Experiments:

Cells were transfected by electroporation (185) using ImF capacitor array charged at 375V for HepG2 cells and 400V for a23 cells. For each transfection, approximately 10^7 cells were mixed with control and test plasmid DNA in 1 ml of RPMI 1640. α_1 globin plasmids were used at 50μg/ml, pSVGH plasmids were used at 10 μg/ml. After transfection the cell suspension was divided into two aliquots for parallel 16 hr normoxic and hypoxic incubations. Normoxic incubations contained humidified air with 5% CO_2. Hypoxic incubation, commencing 1 hr after electroporation was in 1% 0_2 with 5% CO_2 and 94% N_2 in a Napco 7100 incubator.

4.2.4 RNA Analysis:

RNA was prepared using acid/guanidinium thiocyanate/phenol/chloroform extraction method (RNAzol B, Biogenesis Ltd., Bournemouth). Extracted RNA was assayed using RNAse protection assay. ^{32}P labelled riboprobes were produced using SP6 RNA polymerase (186). The growth hormone riboprobe, crossed the boundaries of exon 3 and protected the entire 117 nucleotide sequence of that exon. The α_1 globin riboprobe crossed the cap site of the gene; different probes which protected either 97 or 132 nucleotides of exon 1, were used when the α_1 globin reporter gene was used as the test plasmid, or the control plasmid, respectively.

In all assays, 3-20 μg of total RNA was subjected to double hybridisation with riboprobes for α_1 globin and growth hormone. Fragments protected were separated on 5% polyacrylamide gels and quantified by scintillation counting of excised portions of the dried gel using an LKB flat-bed scintillation counter (Pharmacia-Wallac, Turku, Finland).

Each data point reported is the mean of at least three independent transfection experiments.

4.2.5 Nuclear extract preparation:

Nuclear extracts were prepared using a protocol derived from those of Dignam et al (187) and Kamakaka et al (188). In outline, after parallel normoxic and hypoxic incubations, 1-5 x 10^7 cells were harvested in phosphate buffered saline with EDTA and resuspended in hypotonic buffer (10mM NaHEPES, 5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.1mM EDTA and 0.2mM phenylmethylsulfonyl fluroride (PMSF)). Cells were then lysed by brief ultrasonification (10 Watts for 0.5 seconds, Soniprobe, Lucas Dawe Ultrasonics, London), such that 85% of cells took up trypan blue. The nuclear pellet was mixed with an equal volume of extraction buffer (27mM NaHEPES, 10mM $MgCl_2$, 300mM KCl, 3.2mMDTT, 0.1mM EDTA, 1mM benzamidine, 0.2mM PMSF, 100mM NaF, 1.33 μg/ml aprotinin, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin, 0.2 μg/ml bestatin and 16% glycerol) and agitated gently for 20 minutes at 4^0C. The mix was centrifuged at 100000g for 30 minutes, the pellet and the upper lipid layer were discarded and the clear supernatant, normally containing 6-10 μg/μl of protein, used directly as a nuclear extract or stored in ALIQUOTS in liquid nitrogen.

4.2.6 Dnasel Protection Assays:

Restriction fragments containing the Epo enchancer region were selected such that either end could be end-labelled by DNA polymerase I (Klenow fragment) in the presence of [α-^{32}P]dCTP and used as probes. The probe (1.5 x 10^4 cpm), approximately 50 μg of nuclear extract, and 0.5 μg of poly (dI/dC) were incubated on ice for 15 minutes in 6% glycerol. The final concentrations of reagents in the reaction were 25 mM NaHEPES, 1.0mM EDTA, 3.5mM $MgCl_2$, 30 mM KCl, 10 mM NaF, 0.7 mM DTT, 0.25mM PMSF, 5 mM benzamidine, 10mM glycerophosphate, 2 mM levamisole, 0.1 μg/ml aprotinin. Various amounts of DNaseI (type IV from bovine pancreas, Sigma) diluted in 10mM TRIS pH 8, were then added with $CaCl_2$ (2mM final concentration).

After a 2 minute incubation on ice, 500µl of stop solution (0.11% SDS, 5.5 mM EDTA, and 333 mM NaCl) was added. Samples were extracted with phenol/chloroform and chloroform, precipitated in ethanol, and washed with 80% ethanol. Pellets were redissolved in 80% formamide running buffer and separated by denaturing PAGE (5% acrylamide) prior to autoradiography.

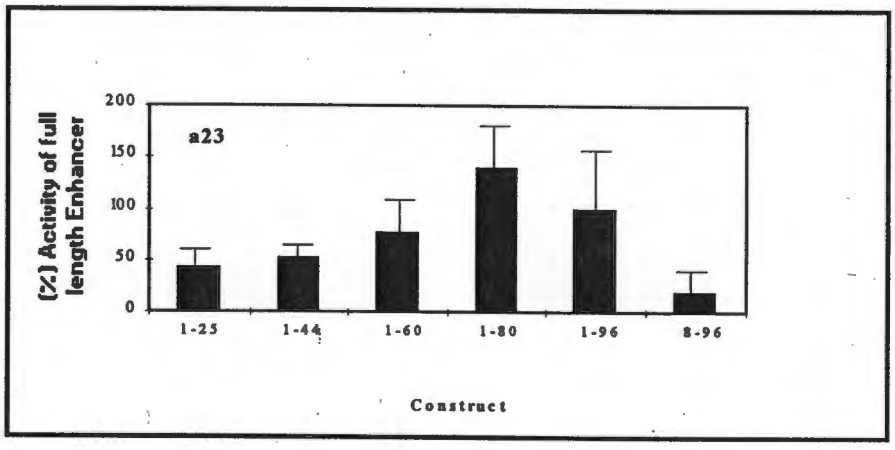
4.3 RESULTS:

4.3.1 Deletion Analysis of the Enhancer in Different Cell Lines:

The minimal enhancer sequence required for activity in different cell lines was determined by deletion analysis. Results are shown in Figure 4.3. In both HepG2 and a23 cells active enhancer sequence was sharply delineated close to the A*pa*I site at the 5' end of the enhancer, but at the 3' end the boundary of active sequence was less clear. In contrast to the HepG2 cells, the sequence between 1-80 appeared to be more active (150% of the full length enhancer) than sequence 1-96 in the a23 cells and a greater proportion of the full length enhancer activity was retained in the shorter 5' sequences.

4.3.2 Mutation Analysis of the Enhancer Region:

To identify critical regions within the enhancer, and to compare these sites in different cell lines, systematic mutations were made in the 5' region of the enhancer which was necessary for function in both HepG2 and a23 cell lines. Thus, 12 consecutive four base pair mutations were made in 48 bases lying 3' to the A*pa*I site (Figure 4.4a,b). In each plasmid a 153bp B*gl*II- P*vu*II restriction fragment containing the mutated enhancer was ligated into the polylinker sequence of pBSα- lying 1.4 Kb 5' to the α_1 globin reporter. Plasmids containing wild type or mutated sequences were then transiently transfected into HepG2 and a23 cells.



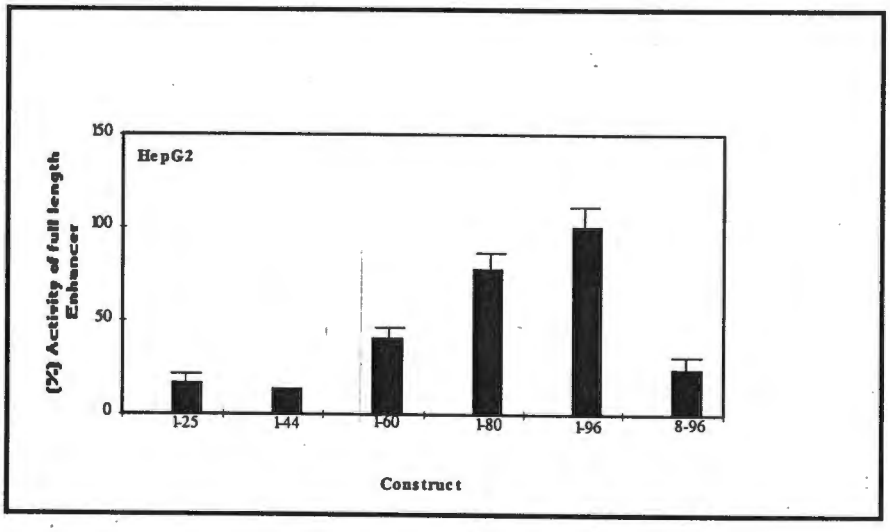


Figure 4.3: Effects of deletions on enhancer function in HepG2 cells and a23 cells. To permit direct comparison of function, plasmids containing the truncated enhancer sequences specified, no enhancer or the full length enhancer were transfected in parallel into aliquots of cells from the same pool. Transfected cells were divided for parallel normoxic and hypoxic incubation. In each transfection, expression of the test plasmids was related to a co-transfected control plasmid (FGH). The results shown are the mean±SD of at least three independent transfections. No enhancer activity was observed in normoxic cells. The degree of hypoxic induction conferred by truncated sequences from the enhancer is expressed as a percentage of the full length enhancer in each set of experiments. In both HepG2 cells and a23 cells deletions of nucleotides 1-25 severely reduced the enhancer function. More gradual loss of function was observed with deletions at the 3' end, where the effect of deletions differed between the cell types.

In each transfection experiment, a single pool of cells was split to permit parallel transfections with plasmids containing no enhancer, the wild type enhancer and the mutated enhancers. After transfection, the cells were split for parallel normoxic and hypoxic incubations. This made it possible to compare directly the level of induction conveyed by wild type and mutated enhancer sequence (Figure 4.4). In these transfections, no constitutive action of the enhancer was observed in either a23 cells or HepG2 cells. Inducible activity of the wild type enhancer was greater in HepG2 than in a23 cells (8-17 fold compared with 3-5 fold), but the effect of mutations was very similar. In each cell type, nucleotide substitutions in three regions corresponding to the mutations in plasmids 2,3,6,9,10 and 11 resulted in severe reduction of enhancer function. In contrast, mutations in plasmids 4,5 and 7 had little effect on activity in either cell type.

4.3.3 DNaseI protection analysis of the enhancer sequences:

To analyse further the structure of the enhancer, DNaseI protection assays were performed using nuclear proteins extracted from HepG2 and a23 cells following parallel incubation under normoxia and hypoxia.

Figure 4.5 shows the results obtained using a probe labelled 5' to the functionally defined sequence. Using nuclear extracts from HepG2 cells, striking protection of nucleotides 28 to 49 was observed. This area was also protected when nuclear extract from a23 cells was used, although the protected region extended further 3' and covered nucleotides 28 to 66. In both cell types a hypersensitive site was observed at nucleotide 23. Another area of protection was observed in the 5' region of the enhancer covering nucleotide -11 to 9 relative to the *Apa*I site. When equal concentrations of extracts from normoxically and hypoxically incubated cells of each type were compared no change in the extent or degree of DNaseI protection was seen.

4.3.4 Functional Analysis of subsequences:

Since subsequences within enhancer may operate independently when placed close to a promotor or when re-iterated, further expression studies were performed using such elements placed immediately 5' to the SV40 promotor. First, the operation of elements consisting of nucleotides 1-25 and 25-60 was compared. Nucleotides 1-25 contain the two critical regions defined by mutants 2,3 and 6, whereas nucleotides 25-60 contain a direct repeat sequence TGACCTCTTGACCC resembling a steroid/thyroid receptor binding element and defined as functionally important by mutants 9-11 (Figure 4.4). When expressed in HepG2 cells, hypoxic expression was induced with the plasmids containing sequence 1-25 adjacent to the SV40 promoter but not with those containing sequence 25-60. This difference was most marked when concatamers of these sequences were compared (Figure 4.6). Re-iteration of sequence 1-25 resulted in large increases in the amplitude of hypoxic induction, whilst expression in hypoxic HepG2 exceeding that of control plasmid containing the SV40 promoter alone by a factor approaching 100 fold. In contrast, re-iterations of sequence 25-60 produced no inducible expression in hypoxic HepG2 cells (Figure 4.6).

When these plasmids were expressed in a23 cells, the pattern of expression was very similar. Activity resided in sequence 1-25 but not in sequence 25-60, although the level of induction was not as great as that observed in HepG2 cells (Figure 4.6).

Also of interest is comparison of sequence 1-60 with sequence 1-25 when placed adjacent to the SV40 promoter. In both HepG2 and a23 cells there was an increase in inducible activity of sequence 1-60 when compared with sequence 1-25.

```
 1          3          5          7          9          11
TTTA       ATGT       AGAT       TAAA       TCAA       TCAA
GGGCCCTACGTGCTGCCTCGCATGGCCCGGCTGACCTCTTGACCCCTC
     AAGC       AGTA       ACGT       TTAG       GAGG       AAGA
       2          4          6          8          10         12
```

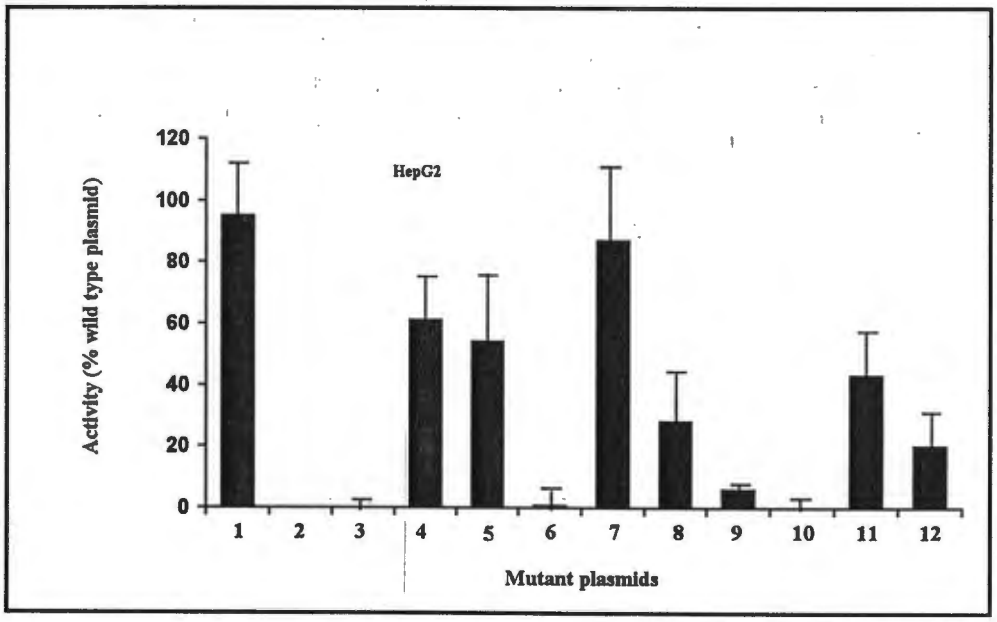


Figure 4.4a

```
  1        3        5        7        9        11
TTTA     ATGT     AGAT     TAAA     TCAA     TCAA
GGGCCCTACGTGCTGCCTCGCATGGCCCGGCTGACCTCTTGACCCCTC
    AAGC     AGTA     ACGT     TTAG     GAGG     AAGA
     2        4        6        8        10       12
```

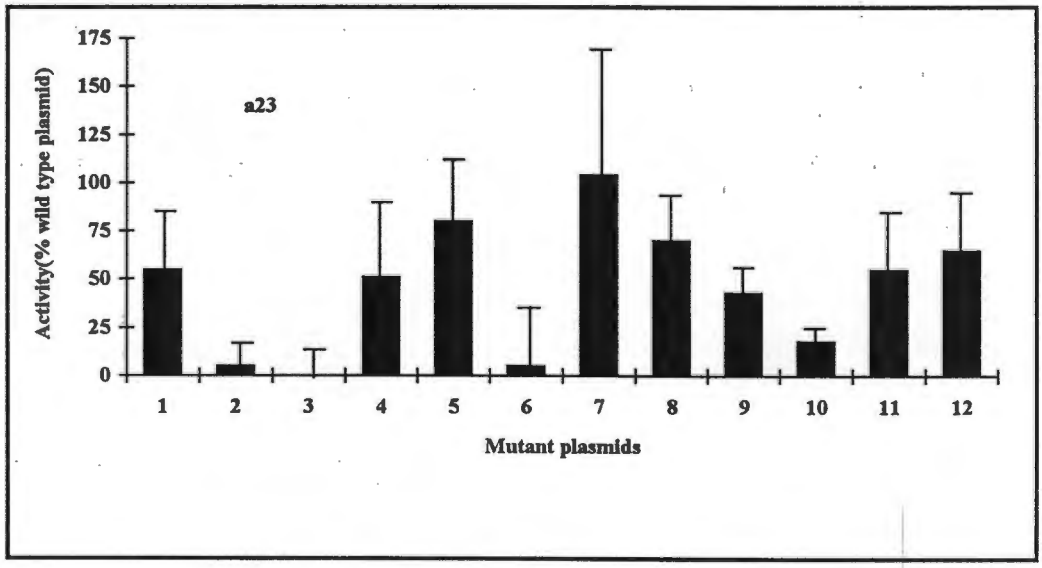


Figure 4.4b

Figure 4.4.a,b: Mutational analysis of the mouse Epo enhancer. Mutations define 3 critical regions in nucleotides 1-48 of the mouse erythropoietin enhancer. Mutations were made in a 153 bp B*gl*II-P*vu*II fragment from the mouse erythropoietin enhancer locus which was placed 1.4 kb from the α_1 globin promoter (see Figure 4.2) Nucleotide substitutions are indicated. Plasmids containing the enhancer mutations, no enhancer or the unmutated enhancer were transfected in parallel into aliquots of cells from the same pool. Transfected cells were divided for parallel normoxic and hypoxic incubation. No enhancer activity was observed in normoxic cells. Hypoxia inducible activity of the mutant enhancers is expressed as a % of that of the unmutated enhancer. Data shown are the mean±SD of three independent experiments. Mutations have similar effects on function in HepG2 cells and a23 cells. In each cell type mutations of nucleotide 5-12, 21-24 and 33-24 caused severe reduction, or complete ablation, of function. In HepG2 cells inducible activity of the unmutated enhancer was greater than in a23 cells, values varying from 8-17 and 3-5, respectively.

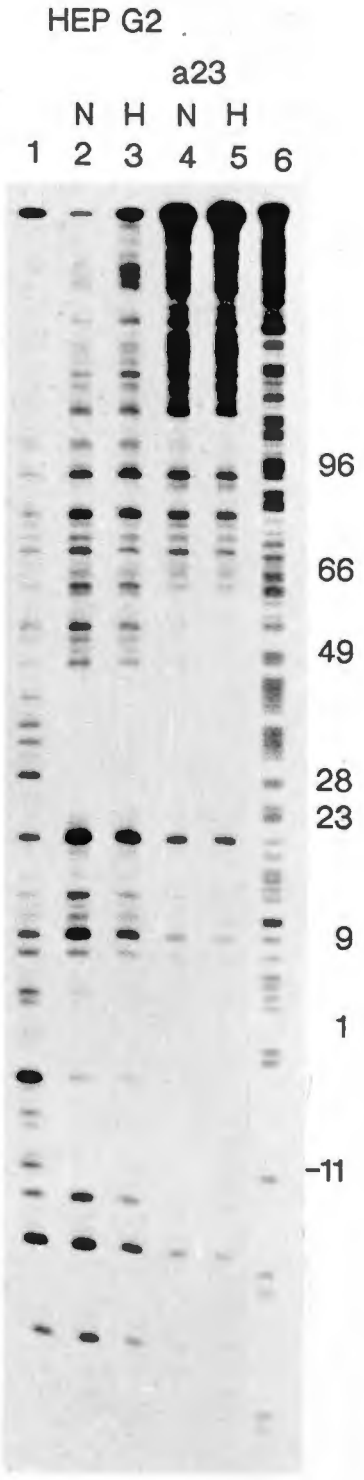


Figure 4.5 DNAseI protection analysis of the mouse erythropoietin enhancer. The 5' end-labelled probe was a 484 bp *Bgl*II-*Sph*I fragment containing the mouse erythropoietin enhancer linked to polylinker sequence from pBluescript SKII. Nuclear extract was prepared after parallel 16 h incubations of cells in normoxia, 21% O_2 (N) and hypoxia, 1% O_2 (H). Lane 1, no extract; lanes 2 and 3, 50 μg of extract from normoxic and hypoxic HepG2 cells; lanes 4 and 5, 50 μg of extract from normoxic and hypoxic a23 cells. Lane 6' is a Maxam and Gilbert G + A column from the same probe. The regions protected are indicated using the numbering system shown in Fig. 4.2

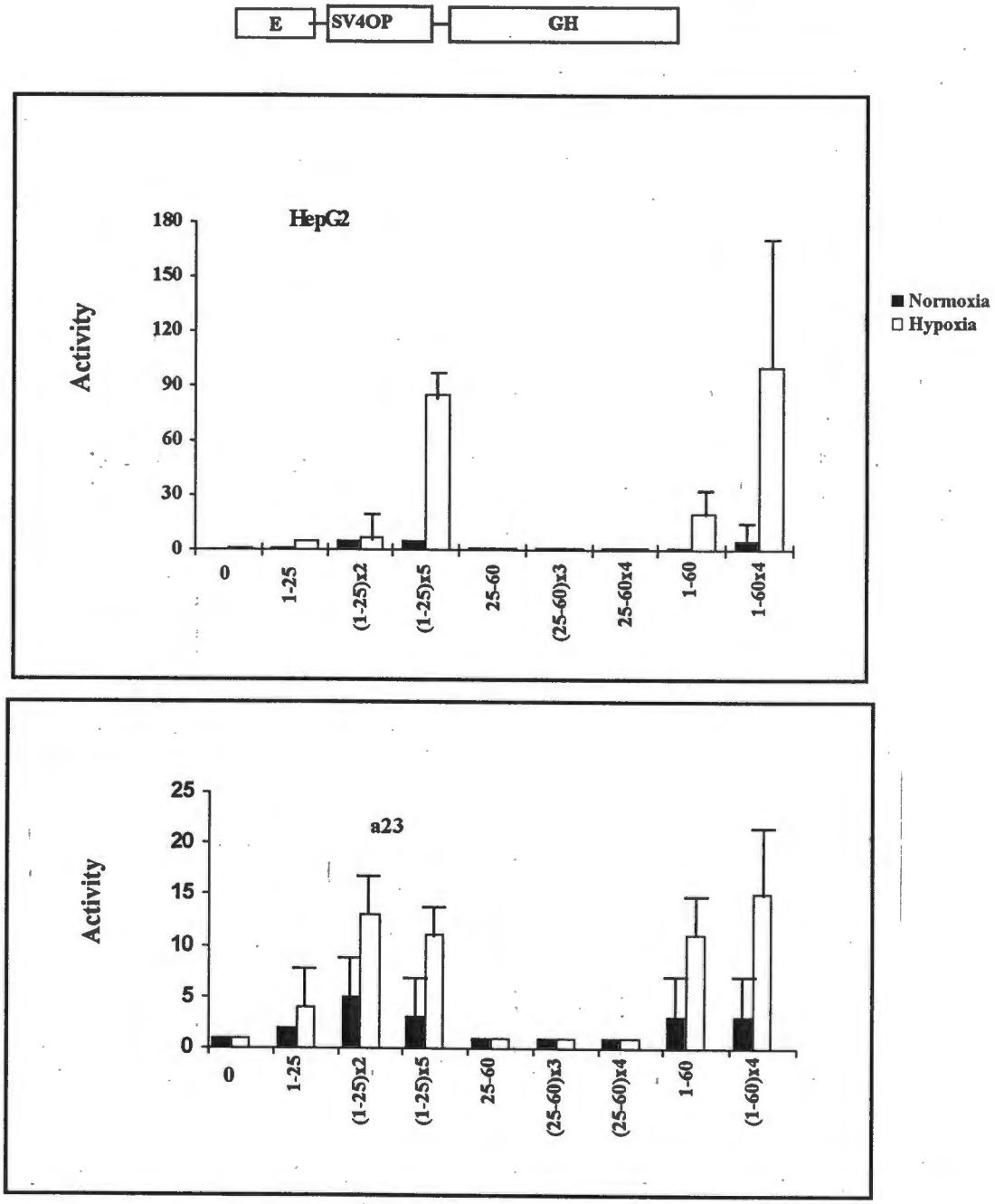


Figure 4.6 Action of subsequences from the mouse erythropoietin enhancer on an adjacent SV40 promoter. The structure of the test plasmids is shown in (A), where E represents erythropoietin enhancer sequence, SV40P represents the SV40 early promoter and GH represent the body of the growth hormone gene. The erythropoietin enhancer sequence fragment (E) used in each test plasmid is indicated beneath the corresponding bars of the histogram and is numbered as in Fig 4.2. A co-transfected plasmid containing the α_1 globin gene alone (pBSα^-) was used to correct for transfection efficiency. Expression is normalised to that of the enhancerless pSVGH in normoxic cells and represents the mean±SD of three independent experiments (B). Inducible activity was conveyed by sequence (25-60) in any cell type. Concatamers of this sequence were tested in either orientation $(25\text{-}60)_3$ and $(60\text{-}25)_4$ but none had any action. The monomeric sequence, 1-60, was more active than the monomeric sequence, 1-25 in both HepG2 and a23 cells.

Since mutation analysis had indicated the existence of two critical regions within the sequence 1-25, short sequences including each of these sites were inserted either as monomers or multimers adjacent to the SV40 promoter and expressed in HepG2 cells. The two sequences 4-15 and 16-27 were also rejoined using an 8bp spacer, (4-15) TCTAGTCC (16-27). None of these sequences conveyed activity when expressed in HepG2 cells (Table 4.1)

Table 4.1 Activity of short sequences either as monomers or multimers within the critical regions of the Epo enhancer. The sequences were inserted adjacent to the SV40 promoter and expressed in HepG2 cells. None of these sequences displayed activity.

CONSTRUCT	CONSTITUTIVE	HYPOXIC/NORMOXIC
Control	1.00	1.46
(4-15)	1.04	1.41
(4-15) x 2	0.69	1.40
(4-15) x 4	0.76	1.31
(4-15) (15-4)	0.76	1.39
(15-4) x 2	0.92	1.24
(27-16) x 2	0.92	1.24
(16-27) x 5	0.82	1.49
(4-15)N8(16-27)	1.20	0.92

4.3.5 Effect of distance from a promoter:

While both deletion and mutation analysis in HepG2 cells indicated that sequences 3' to nucleotide 25 were obligatory for enhancer function when placed 1.4 kb from the α_1 globin promoter, these sequences were not obligatory and had no activity when placed adjacent to an SV40 promoter. To distinguish whether this difference was a function of distance from the promoter or some specific aspect of the promoter sequence, expression of plasmids containing subsequence 1-25 or subsequence 1-96 approximately 0.1 and 1.4 kb from the α_1 globin promoter was compared. In HepG2 cells, sequence 1-96 was almost equally active in both positions whereas subsequence 1-25 was only active close to the promoter (Figure 4.7).

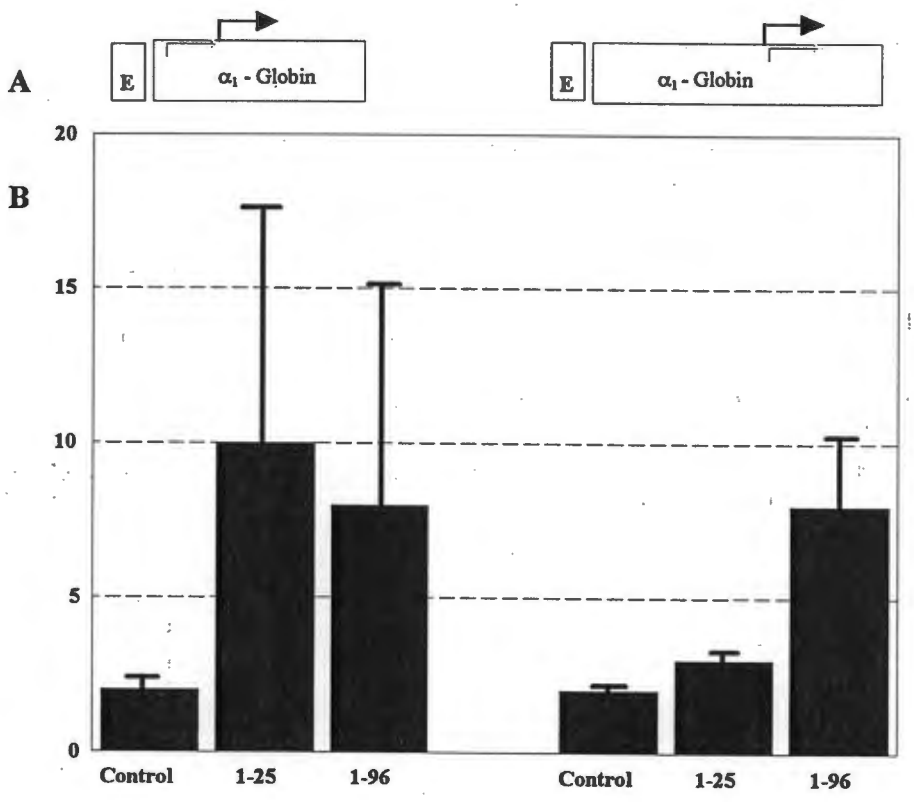


Figure 4.7 Effect of distance from a promoter on function of the mouse erythropoietin enhancer. The structure of the test plasmids which were used in these experiments are shown (A). The pairs of plasmids were identical except that one contained 124 bp 5' flanking sequence from the α_1 globin gene and the other contained 1.4 kb of this sequence separating the enhancer from the α_1 globin promoter. A plasmid (FGH) was co-transfected to control for variations in transfection efficiency, RNA synthesis and RNA recovery. Transfected cell were divided for parallel normoxic and hypoxic incubation. (B) Normoxic and hypoxic expression of the test plasmid were normalised with respect to expression of the control plasmid. The values plotted represent the ratio of normalised hypoxic to normoxic expression from three separate transfections (mean±SD). In HepG2 cells nucleotides 25-96 were necessary for enhancer function at a distance from the α_1 globin promoter but not when the enhancer was close to the promoter.

4.4 Discussion

Deletion of sequence close to the ApaI site at the 5' end of the mouse Epo enhancer (construct 8-96, Figure 4.3) sharply reduced enhancer activity indicating that these 88 nucleotides contained bases critical for inducible enhancer operation. However the length of the minimal functional enhancer indicated that enhancer function was most probably dependent on several other critical sequences. This was borne out by mutational analysis of the first 48 nucleotides (Figure 4.4) which defined three critical sites corresponding to nucleotides 5-12, 21-24 and 33-44. The sequences represented by 5-12 and 21-24 do not have homology with other known transcription factor binding motifs. Mutations affecting nucleotides 33-44 disrupt the sequence TGACCTCTTGACCC which may represent a binding site for an orphan receptor of the steroid/thyroid hormone family (120).

The constructs used in these experiments contained the enhancer element separated from the α_1 globin promoter by 1.4 kb. All 3 regions within the enhancer were necessary for function in this setting. It has been shown that isolated sequences within enhancers which are inactive when placed at a distance form a promoter may operate when re-iterated or placed close to the promoter (183,189,190). To test the subsequences individually for their ability to convey inducible responses we therefore placed monomers or concatamers of each element adjacent to the SV40 promoter.

Concatamers of nucleotides 1-25, which contained the 5' critical sites, but neither of the TGACCC/T half sites, were compared directly with concatamers of nucleotides 25-60, containing the TGACCC/T direct repeat. The behaviour of the two regions was quite different. Concatamers of nucleotides 1-25 were highly active and showed inducible activity which was substantially greater than even the full length enhancer. In contrast, neither monomers nor concatamers of nucleotides 25-60 showed any inducible activity.

The independent operation of nucleotides 1-25 in the pSVGH plasmids was confirmed by the inducible operation of this sequence in pBSΔα 1-25, which contained a different heterologous promoter and reporter gene. The two critical subsequences within this region indicated by mutation of nucleotides 5-12 and 21-24 did not appear to function independently since a variety of concatamers of nucleotides 4-15 or 15-27 were not functional. Furthermore, introduction of spacing nucleotides TCTAGTCC in construct 4-15(N8)16-27 ablated function. Thus, this study defines the inducible operation of a 25 nucleotide sequence containing two sites which are critical for function.

Studies on the human Epo enhancer, have shown that mutations in two regions of the enhancer can destroy function (119,120). The mutated region of the mouse Epo enhancer was somewhat more extensive and covered the regions that are homologous to both of those examined in the human studies.

Despite the use of a different system of nucleotide substitution, the results are entirely consistent with the experimental observations in each of the studies of the human enhancer. The critical regions defined by mutations 2-3 and 6 correspond to sequences designated BS-1 and BS-2 in one study (119) whereas mutations 9-11 affect the directly repeated TGACCC/T half sites which were shown to be critical for function in the other study (120).Thus at least 3 functionally critical regions have been identified in murine and human Epo enhancers that control Epo gene expression.

Different conclusions were reached in the two studies on the human enhancer as to the function of each region. Blanchard *et al* (120) argued that the directly repeated half-sites were the critical site of interaction, and postulated that modification of a factor at this site mediated the oxygen regulated response. In contrast, Semenza and Wang (119) demonstrated an inducible DNA binding

activity using an oligonucleotide from the 5' region of the enhancer, and found a 33bp oligonucleotide extending to include only 4bp of one of the directly repeated half-sites retained some inducible activity which could be restored to the level shown by the full length enhancer by re-iteration. They postulated that the inducible interaction took place with sites at the 5' end of the enhancer with the direct repeat element functioning to amplify the response. This study supports the latter explanation in that concatamerization of the subsequence 1-25 resulted in a marked increase in activity.

When the non-erythropoietin producing a23 cells are compared with HepG2 cells the same two critical sites were necessary for function in the 5' region of the enhancer. Furthermore, in both HepG2 and a23 cells, sequences lying 3' to this region were important for operation at a distance, amplified the response when the enhancer sequence was close to the promoter, but did not function independently. Maxwell et al (121) showed that inducible operation of the mouse Epo enhancer in Epo non-producing cell lines was distinct from other cell stress responses such as heat chock, and resembled the inducible response of the native Epo gene in hepatoma cells. They argued that a similar or identical mechanism of oxygen sensing and signal transduction must operate widely, most probably inducing the expression of other genes in non-Epo producing cells. Such a system could induce gene expression by activation of a variety of transcription factors which might vary between different cell types and recognise different motifs in cis-acting regulatory sequences (182,183). However, in this study, the demonstration that similar subsequences within the Epo enhancer were critical for function in different cell lines strongly suggest that common factors are operating this response in different cell lines.

In the DNAseI protection assays inducible changes were not observed in protection of the critical sequences in the 5' region of the murine enhancer and at similar sites in recent analysis of the human Epo enhancer (119).

However, using electrophoretic mobility shift assays, Semenza and Wang have observed inducible binding of a nuclear factor from Hep3B cells, which they designated HIF-1 (hypoxia inducible factor), to nucleotides 3-21 of the human enhancer (119). In this study, the results are consistent with the binding of such a factor to the mouse Epo enhancer and strongly suggest that it operates in erythropoietin non-producing cell lines. This DNA binding protein has now been characterized (191). It is made up of four polypeptides composed of two different subunits: 120kda HIF-1 alpha and 91-94kda HIF-1 beta. HIF-1 was shown to bind specifically to the wild type of the Epo enhancer in Hep3B cells but not to the mutant sequence that lacks hypoxia-inducible enhancer activity. It was also shown to operate in the non-Epo producing HeLa S3 cells suggesting a general role of HIF-1 in hypoxia signal transduction and transcriptional regulation.

Using nuclear extract from HepG2 cells in DNAseI footprinting studies, striking non-inducible protection of nucleotides 29-59, which include the TGACCC/T repeat element, was observed. This footprint was closely similar to that described on the human enhancer using extract from Hep3B cells (119,120). However, a larger footprint extending over a further 18 nucleotides 3' to this region was observed in a23. Although this indicates differences in the DNA-protein interactions with the 3' end of the enhancer it does not fully explain the functional data. For instance, deletion analysis in HepG2 cells implicated functionally important sequences lying 3' to nucleotides 50 which were necessary for enhancer operation at a distance, but which were not protected. It therefore appears that, in addition to the TGACCC/T direct repeat, further

subsequences in the 3' region of the enhancer modulate the function of the inducible element. Precise definition of these sequences and their interactions will require further work.

Based on responses obtained by exposure of hepatoma cells to transition elements, haem synthesis inhibitors, and haem protein ligands such as carbon monoxide, Goldberg et al (101) proposed that the oxygen sensor controlling Epo production by hepatoma cells might be a haem protein. Recently, Yoshioka showed that nitric oxide synthase (NOS) itself has a haem moiety in its structure. Furthermore, NOS is stimulated by TNFα and NO may be one mediator of cytokine-induced haematopoietic suppression (180); the NO/guanosine 3'5' cyclic monophosphate system plays an important role in oxygen sensing and hypoxic regulation of Epo production (109). Thus it can be postulated that in an inflammatory/infective process where both TNFα and NO are released, TNFα may disrupt the interaction of transcription factors with critical sites on the enhancer element thereby inhibiting to some extent the activation of transcription of the Epo gene by NO. Further work is required to examine this postulate.

CHAPTER 5

Overall Conclusions

Tuberculosis is acquiring increasing importance throughout the world especially with the advent of human immunodeficiency virus (HIV) infections. In South Africa, tuberculosis is a major cause of morbidity and mortality.

The anaemia associated with the disease contributes greatly to the morbidity. The anaemia can be categorised biochemically as anaemia of chronic disorders in that serum iron is low, the transferrin is decreased and iron stores are adequate or raised. Understanding the pathophysiology of this anaemia is important in that it would explain how inflammation causes anaemia and perhaps therapeutic options or avenues by which this anaemia can be treated.

The anaemia of pulmonary tuberculosis is associated with a disturbance of iron metabolism and erythropoietin production. These findings are in agreement with that of the anaemia associated with rheumatoid arthritis (146), cancer (148), HIV (149) and Crohn's disease (163). In this study (Chapter 2), it was clearly shown that anaemia develops in pulmonary tuberculosis as a result of the degree of inflammation since the level of C-reactive protein was significantly higher in the anaemic compared to the non-anaemic PTB group. Furthermore, with anti-tuberculous therapy, there was a significant decline in the level of CRP in the anaemic PTB group which resulted in normalization of the haemoglobin and iron levels within three months of initiation of therapy. For the same degree of anaemia, patients with pure iron deficiency anaemia mounted a significantly greater Epo response compared to the anaemic PTB patients. It appears, therefore, that PTB-related inflammation releases a substance or substances that interfere with the production of Epo.

To investigate these findings further, measurements of production of the endogenous cytokine TNFα from PBMC's of patients with PTB (anaemic and non-anaemic) and iron deficiency anaemia were undertaken (Chapter 3).

Patients in the anaemic PTB group produced significantly higher levels of TNFα compared to patients in the non-anaemic PTB and iron deficiency groups. These findings correlated well with the fact that the anaemic PTB group had the greatest degree of inflammation as shown by the higher CRP level. To determine the effect of TNFα on Epo production, the monocyte supernatants from the the three groups were added to HepG2 cells in culture. The production of Epo by these cells were markedly inhibited by the supernatants from the anaemic PTB group. Furthermore, this inhibition could be reversed by the use of anti-TNFα-specific antibodies. These findings are in agreement with that of Faquin et al (152). Jelkmann et al (153). Unlike these studies, however, we employed endogenously produced TNFα rather than a recombinant TNFα and we clearly showed reversal of inhibition with the anti-TNFα antibodies.

In anaemic f PTB there was an inverse relationship between the Hb level and Epo production (Chapter 2). In iron deficiency anaemia, however, for the same degree of anaemia, the production of Epo was markedly increased compared to that of PTB. This suggested that hypoxia as a result of anaemia in PTB did not result in an adequate Epo response perhaps due to TNFα interferance with the oxygen sensing mechanism. TNFα is known to stimulate the production of NO (108). In inflammatory/infective states both NO and TNFα are released and it has been shown that the NO/cGMP system play an important role in oxygen sensing and hypoxic regulation of Epo production (111). In an attempt to study the oxygen sensing mechanism further, it was decided to investigate the regulation of the Epo gene.

Tranfection studies in HepG2 and Hep3B cells have defined an oxygen regulated enhancer commencing 120 bp 3' to the polyA addition site of the human and mouse Epo genes (116-120). In this study (Chapter 4) the enhancer element was subjected to mutational analysis in an attempt to define the critical sites on the enhancer involved in oxygen sensing. In agreement with Pugh et al (118), Semenza et al (119) and Blanchard

et al (120), we have identified a 25 nucleotide enhancer element in the 3'-untranslated region of the mouse Epo gene which confired oxygen sensing in both Epo and non-Epo producing cell lines. The enhancer element can be divided into three functionally important domains (Figure 5.1).

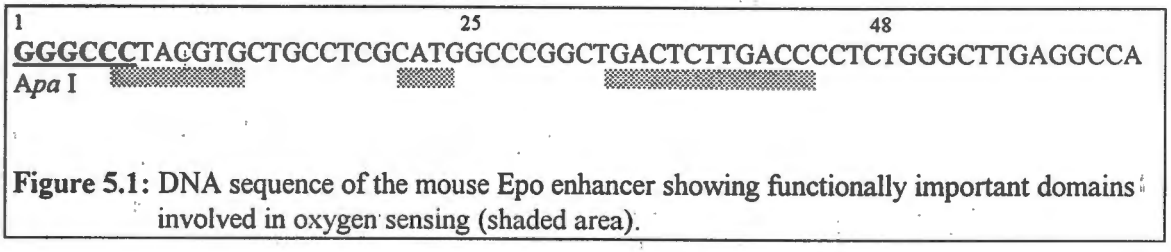


Figure 5.1: DNA sequence of the mouse Epo enhancer showing functionally important domains involved in oxygen sensing (shaded area).

Mutation of these three domains resulted in ablation of the oxygen sensing capacity of the enhancer element. An explanation for this ablation of activity could be due to binding of transcriptional repressors to these critical sites. DNase footprint analysis revealed that although transcription factors bound to these regions (Figures 4.5 & 5.2), there was no difference between normoxia and hypoxia (Figure 4.5), thus ruling out the possibility of altered transcription factor binding.

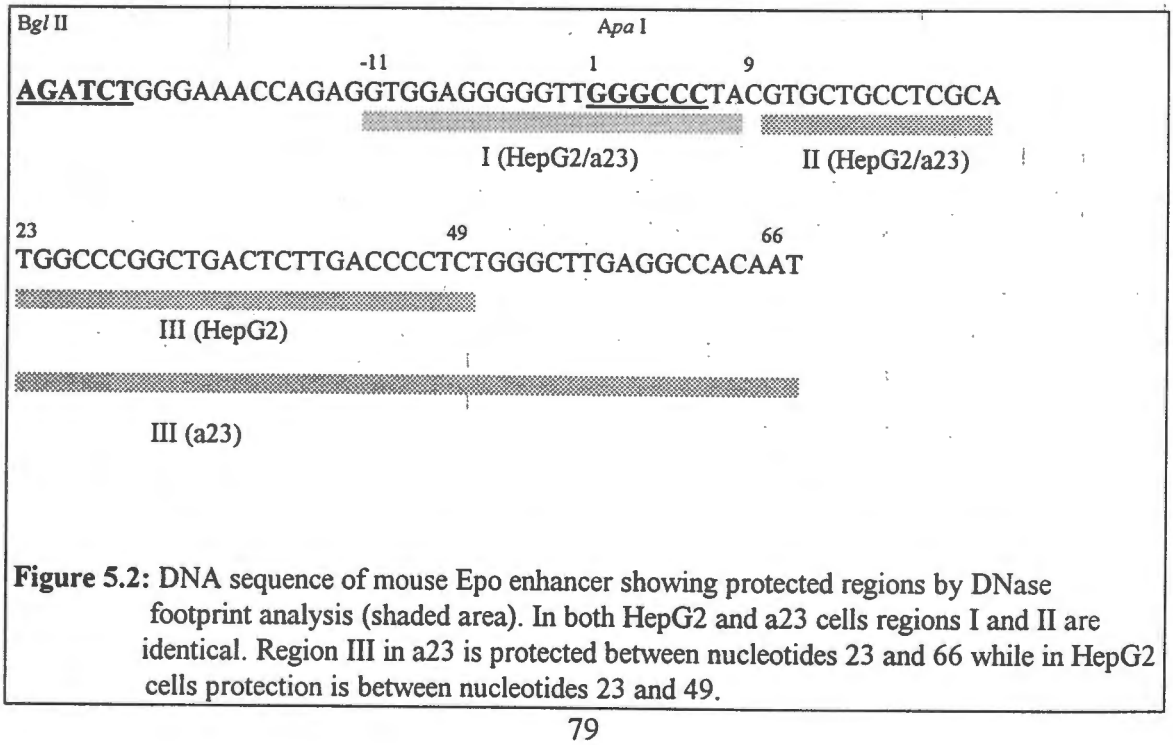


Figure 5.2: DNA sequence of mouse Epo enhancer showing protected regions by DNase footprint analysis (shaded area). In both HepG2 and a23 cells regions I and II are identical. Region III in a23 is protected between nucleotides 23 and 66 while in HepG2 cells protection is between nucleotides 23 and 49.

It is conceivable, however, that different proteins with different functional activities bind to the same element, thereby changing the transcriptional activity of the gene. Further proof of this will only come from experiments attempted at purifying these transcription factors. Semenza and Wang isolated a nuclear factor from Hep3B cells (HIF I) which they showed binds to nucleotides 3-21 of the human Epo enhancer (119). This factor binds to the wild type of the enhancer but not to the mutated sequence (191). It was also shown to be present in the non-Epo producing HeLa S3 cells (191). The results obtained in this study, are consistent with the binding of such a factor to the mouse Epo enhancer and strongly suggest that it operates in the non-Epo producing a23 cells.

TNFα could inhibit Epo production in one of (or a combination of) different pathways. One such mechanism could be that TNFα inhibits the production of a transcription factor that bind to these elements. Alternatively, it could stimulate the production of a negative transcription factor, such as that reported by Beru et al (113). A more likely explanation for action of TNFα could be modification of transcription factors via the activation of signal transduction pathways for example phosphorylation of transcription factor is known to affect DNA binding activity (192).

APPENDIX 1

DIAGNOSTIC CRITERIA FOR PULMONARY TUBERCULOSIS

DIAGNOSTIC CRITERIA FOR PULMONARY TUBERCULOSIS

Category	Subcategory	Score
Chest Radiograph	1. Lesion (s) in upper or lower lobe apical segment (s):	
	A. Infiltration or scarring with cavitation	5
	B. Non-confluent infiltration or scarring without cavitation	3
	2. Lesion (s) elsewhere in the lung (not apico-posterior)	
	A. with cavitation	2
	B. without cavitation	1
	3. Diffuse lung lesions:	
	A. Miliary	3
	B. Non-Miliary	
	4. Pleural lesion (s)	
	A. Pleural effusion (s)	3
	5. Lesion (s) in upper or lower lobe apical segment which is new or enlarging and shows no sign of resolution on a 2-month follow-up radiograph	5
Sputum	1. Direct positive for AFB's:	
	A. Once	3
	B. Twice	7
	C. 3 times	10
	2. Culture positive for *Mycobacterium tuberculosis*	
	A. Once	7
	B. Twice	10
Tuberculin Test	1. Heaf grade 3	2
	2. Heaf grade 4	4
Histology	1. Lung, liver or lymph node	
	A. Epitheloid/giant cell granuloma	5
	B. Granuloma with necrosis	7
	C. Granuloma with AFB's	10
	2. Pleura	
	A. Epitheloid giant cell granuloma	10
Therapeutic Clinical Trial	1. Radiological improvement after 2 months of treatment as compared with the chest radiograph after 1 month of treatment.	3

NOTE: A total score of **10** is required for the diagnosis of active pulmonary tuberculosis, but <u>only</u> **1** score may be used from each category.

APPENDIX 2

BIOCHEMICAL AND HAEMATOLOGICAL PARAMETERS OF PATIENTS AND HEALTHY VOLUNTEERS

NORMAL RANGES

(According to the Haematology and Biochemical laboratories of Groote Schuur Hospital Cape Town)

Haematology		**Biochemistry**	
Hb (m)	133-173 g/L	Na+	135-145 mmol/L
(f)	116-156 g/L	K+	3.5-5.5 mmol/L
MCV (m)	81-93 fl	Urea	1.7-6.7 mmol/L
(f)	81-95 fl	Cr	75-115 µmol/L
MCH	28-30 pg	S-Ferritin	20-200 µg/L
Hct (m)	40-50 %	RC-Ferritin	0.002-0.02 fg/cell
(f)	35-45 %		
WCC	4-11 $x10^9$/L	S-Iron	9-30 µmol/L
S-Folate	> 2.5 ng/ml	TIBC	45-65
RC-Foltate	230-710 ng/ml	% Sats	20-50
Vit B12	180-710 pg/ml		

PERSONAL AND LABORATORY DETAILS OF ANAEMIC PTB PATIENTS PRIOR TO THERAPY

			BIOCHEMISTRY				HAEMATOLOGY											IRON STUDIES					
Pt No.	Age (yrs)	Gender	Na+ (mmol/L)	K+ (mmol/L)	Urea (mmol/L)	Cr (umol/L)	Hb (g/L)	WCC ($x10^9$/L)	Hct (%)	MCV (fl)	MCH (pg)	Retics (%)	sB12 (pg/ml)	sFolate (ng/ml)	RC Folate (ng/ml)	CRP (mg%)	Epo (mU/ml)	sIron (umol/L)	TIBC	% Sat	sFerritin (ug/L)	RC Ferritin (fg/cell)	E&C Score
1	42	M	132	4.1	3.2	62	105	7.7	30	93	32	1.2	370	2.7	550	5	70	3	39	10	>1000	0.044	12
2	50	M	141	4.7	3.2	79	91	10.3	27	82	27	0.7	780	2	580	9.2	35	6	44	14	>1000	0.038	11
3	38	M	143	4.8	3.6	62	87	6.6	27	94	33	1.5	520	3.9	520	6	68	5	52	10	140	0.024	12
4	29	M	135	4.8	3.9	70	91	11.1	29	74	23	0.8	350	2	250	8.6	37	7	42	17	>1000	0.038	10
5	23	M	134	4.5	4.3	53	95	7.5	31	68	21	0.5	310	2.2	480	7.4	37	6	44	14	>1000	0.039	13
6	31	F	141	5.4	2.9	106	108	13.9	34	76	24	0.4	370	3.5	660	4.8	48	7	46	15	150	0.014	13
7	28	M	142	4.2	5	70	87	11.7	27	84	27	0.9	1400	3.3	560	9.4	25	6	42	14	820	-0.036	12
8	26	F	137	5.4	2.4	94	69	8	21	64	21	0.8	350	2.5	350	9.2	140	5	44	11	220	0.004	10
9	42	M	136	5	3.2	69	104	10.4	35	92	35	0.3	950	9.7	310	8.2	33	7	44	16	>1000	0.037	14
10	28	F	140	4.4	3	82	107	10.4	37	84	37	0.2	1000	3.2	430	9.7	44	4	42	10	130	0.005	10

PERSONAL AND LABORATORY DETAILS OF NON-ANAEMIC PTB PATIENTS PRIOR TO THERAPY

			BIOCHEMISTRY				HAEMATOLOGY											IRON STUDIES					
Pt No.	Age (yrs)	Gender	Na+ (mmol/L)	K+ (mmol/L)	Urea (mmol/L)	Cr (umol/L)	Hb (g/L)	WCC ($x10^9$/L)	Hct (%)	MCV (fl)	MCH (pg)	Retics (%)	sB12 (pg/ml)	sFolate (ng/ml)	RC Folate (ng/ml)	CRP (mg%)	Epo (mU/ml)	sIron (umol/L)	TIBC	% Sat	sFerritin (ug/L)	RC Ferritin (fg/cell)	E&C Score
1	34	M	147	4.2	3.9	76	162	10.8	48	91	31	1	1480	4.4	550	3.3	30	8	48	17	120	0.042	10
2	54	M	135	4.4	4	65	146	6	44	82	27	0.8	560	3.2	320	2.9	14	11	50	22	70	0.04	12
3	40	F	138	3.8	4.2	75	142	7.5	42	96	35	1.2	330	3.4	590	2.4	22	12	52	23	400	0.022	11
4	54	M	140	3.5	4.1	80	135	8.4	47	84	28	0.9	500	2.8	325	2.6	38	24	54	44	320	0.036	12
5	35	M	137	5	3.3	90	153	14.8	41	88	32	0.5	580	2.7	280	3.2	21	15	52	29	290	0.027	10
6	30	M	139	4	3.5	78	148	9	44	86	30	1.2	950	3.2	250	2.6	22	18	50	36	42	0.011	13
7	47	M	140	4.2	5.5	96	151	7.3	43	96	33	0.4	750	2.7	300	2.9	17	10	50	20	110	0.03	11
8	29	F	135	4	3.4	66	142	7.3	43	96	31	0.8	700	3.2	250	5.6	23	14	52	27	60	0.011	12
9	33	M	140	4.2	3.8	78	149	7.1	45	89	29	1	650	4.5	650	4.2	12	16	54	30	310	0.024	12
10	25	F	139	4.8	4.5	76	142	11.6	40	81	28	0.7	900	2.9	320	3.3	13	12	50	24	300	0.022	10

PERSONAL AND LABORATORY DETAILS OF IRON DEFICIENCY ANAEMIC PATIENTS PRIOR TO THERAPY

			BIOCHEMISTRY				HAEMATOLOGY										IRON STUDIES					
Pt No.	Age	Gender	Na+	K+	Urea	Cr	Hb	WCC	Hct	MCV	MCH	Retics	sB12	sFolate	RC Folate	CRP	Epo	sIron	TIBC	% Sat	sFerritin	RC Ferritin	Cause of
	(yrs)		(mmol/L)	(mmol/L)	(mmol/L)	(umol/L)	(g/L)	(x10^9/L)	(%)	(fl)	(pg)	(%)	(pg/ml)	(ng/ml)	(ng/ml)	(mg%)	(mU/ml)	(umol/L)			(ug/L)	(fg/cell)	Anaemia
1	22	F	142	4.8	3.8	79	78	8.2	27	74	18	0.3	315	5.2	307	u/d	495	4	66	6	12	0.01	Menorrhagia
2	25	F	145	4.2	3.5	66	80	6.6	28	76	24	0.2	320	2.6	309	"	166	5	71	7	10	0.004	"
3	30	F	140	3.8	3.8	76	100	5.6	33	62	20	0.5	460	4.3	470	"	68	3	76	4	11	0.009	Blood donor
4	32	F	135	3.8	3.9	77	76	6.4	28	64	21	0.4	460	5.2	258	"	166	3	88	3	9	0.006	Menorrhagia
5	23	F	142	3.5	4	95	86	7.8	28	79	25	0.4	460	4.3	297	"	100	3	72	4	9	0.003	Blood donor
6	40	M	138	4	2.9	60	108	6.9	33	78	26	0.6	480	6.6	304	"	63	4	72	6	10	0.009	"
7	28	M	135	4.5	3.4	78	112	3.9	33	81	26	0.3	470	6.5	324	"	50	5	72	7	8	0.012	"
8	29	M	138	4.7	3.8	80	97	5.3	28	82	25	0.7	450	5	311	"	103	2	78	7	7	0.014	GIT bleed
9	42	M	134	4	3.9	96	92	6.5	27	80	23	0.4	470	6.3	345	"	138	6	70	8	9	0.008	"
10	26	M	135	4.2	4.2	75	115	8.1	30	82	26	0.5	480	5.8	329	"	68	6	70	9	8	0.004	"

PERSONAL AND LABORATORY DETAILS OF HEALTHY VOLUNTEERS

			BIOCHEMISTRY				HAEMATOLOGY											IRON STUDIES				
Pt No.	Age	Gender	Na+	K+	Urea	Cr	Hb	WCC	Hct	MCV	MCH	Retics	sB12	sFolate	RC Folate	CRP	Epo	sIron	TIBC	% Sat	sFerritin	RC Ferritin
	(yrs)		(mmol/L)	(mmol/L)	(mmol/L)	(umol/L)	(g/L)	(x10⁹/L)	(%)	(fl)	(pg)	(%)	(pg/ml)	(ng/ml)	(ng/ml)	(mg%)	(mU/ml)	(umol/L)			(ug/L)	(fg/cell)
1	55	F	140	4.2	4	82	120	6.3	35	94	32	1	500	4	310	u/d	25	10	56	18	28	0.024
2	25	F	145	3.8	3.9	75	123	8.8	37	86	28	0.8	410	6	550	"	24	14	56	25	38	0.026
3	23	F	139	5.2	4.5	66	120	4.2	36	84	29	0.8	550	4.2	340	"	23	14	62	22	30	0.021
4	38	M	138	4.5	5	95	156	9.7	42	92	34	1.2	510	4.8	340	"	21	16	56	29	110	0.02
5	40	M	138	4.2	4.3	98	162	10.4	48	84	28	0.9	410	6	570	"	26	14	52	29	70	0.022
6	24	F	135	4	4	70	148	6.8	44	95	33	1.2	380	5.8	580	"	23	14	60	23	55	0.025
7	23	F	137	3.5	2.9	66	123	7.1	36	95	32	0.8	620	5.5	510	"	24	13	54	24	60	0.021
8	30	M	143	4.8	5	90	146	5.4	38	90	34	0.7	450	6.2	570	"	25	20	58	34	90	0.02
9	23	M	142	4.5	5.2	76	161	8.7	47	88	30	1	700	4.5	520	"	21	17	56	30	80	0.019
10	24	M	140	4	3.8	85	145	5.8	44	94	32	0.9	590	5.8	540	"	17	15	60	25	60	0.024

APPENDIX 3

PEER REVIEWED PAPERS

Blunted Erythropoietin Response to Anaemia in Pulmonary Tuberculosis

All clinical and experimental work elucidated in this paper was done entirely by me.

Characterisation of functional domains within the mouse erythropoietin 3' enhancer conveying oxygen-regulated responses in different cell lines

All experimental work on HepG2 and a23 cells elucidated in this paper were carried out entirely by me. Written permission was obtained from Dr P Ratcliffe, Head of the Erythropoietin Research Group, to publish the work in this thesis.

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Blunted erythropoietin response to anaemia in tuberculosis

Ebrahim O, Folb PI, Robson SC, Jacobs P. Blunted erythropoietin response to anaemia in tuberculosis.
Eur J Haematol 1995: 55: 251–254. © Munksgaard 1995.

Abstract: The precise cause of the anaemia that is commonly associated with severe pulmonary tuberculosis (PTB) has not been elucidated. The role of erythropoietin (Epo), the central hormone regulating red cell formation, still awaits clarification. We therefore determined serum Epo levels in patients with PTB; group 1, haemoglobin less than 110 g/L, group 2, haemoglobin greater than 110 g/L; group 3, controls, consisted of matched individuals with uncomplicated iron deficiency; group 4, healthy volunteers. Peripheral blood monocytes were obtained from patients with PTB and the controls, cultured, and the supernatant fluid (SNF) harvested. Tumour necrosis factor alpha (TNFα) levels were determined in the SNF, which were then added in various dilutions to a hepatocellular carcinoma cell line (HepG2) capable of regulated EPO synthesis *in vitro*. The influence of this cytokine was defined by the addition of specific neutralising anti-TNFα antibodies in this assay system. Patients in group 1 had significantly lower Epo levels (54 ± 11 mU/mL) compared with those in group 3 (142 ± 41 mU/mL) ($p < 0.01$). Monocyte supernatants from patients in the anaemic PTB group had markedly elevated TNFα levels and significantly suppressed Epo output by HepG2 cells *in vitro* ($p < 0.01$). This inhibition was consistently abrogated by anti-TNFα antibodies. Serum Epo levels were inappropriately low in untreated PTB patients when compared with corresponding haemoglobin levels in iron deficient controls. This blunted response could be ascribed to release of TNFα or other cytokines by activated monocytes.

O. Ebrahim [1], P. I. Folb [1], S. C. Robson [2] and P. Jacobs. [3]

Departments of [1] Pharmacology, [3] Haematology, the University of Cape Town Leukaemia Centre and the [2] MRC/UCT Liver Research Centre, Department of Medicine, Groote Schuur Hospital, Observatory, Cape, South Africa

Key words: pulmonary tuberculosis – erythropoietin – anaemia – tumour necrosis factor alpha

Correspondence: Professor Peter Jacobs, MD, PhD, Haematology, Wynberg Hospital, PO Box 18171, Wynberg 7824, Cape Town, South Africa

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The subnormal haemoglobin level associated with severe pulmonary tuberculosis (PTB) is considered to be multifactorial and attributable to the underlying chronic inflammatory disorder (1, 2). Disturbances in iron metabolism (3), decreased red cell survival (4), and reduced erythropoietin (Epo) responses (5) have been described in the pathogenesis of this anaemia. Although both impaired production and response to Epo may contribute to the reduced red cell mass (6–10), a single mechanism has not been identified.

Mycobacterial lipoarabinomannan is a potent trigger of tumour necrosis factor alpha (TNFα) release from monocyte-macrophages (11), with its production in the distinctive granulomata being considered an essential component of the immune response to mycobacterial infections (12), while peripheral blood mononuclear cells (PBMC) from these patients are known to release large quantities of TNFα *in vitro* (13). The fever, weight loss and tissue injury that characterise advanced tuberculosis may be attributable in part of TNFα (14), which also exerts an inhibitory effect on haematopoiesis, both *in vitro* (15) and *in vivo* (16) and suppresses the production of Epo in a human hepatocellular line, HepG2, *in vitro* (17, 18).

In further studying these interrelationships, we have demonstrated suboptimal Epo plasma levels for the degree of anaemia associated with PTB and we present *in vitro* data indicating that mycobacterial infection induces monocyte release of TNFα. On this basis, we advance the hypothesis that increased output of this inhibitory cytokine blunts the synthesis or release of the hormone, so that it is inappropriately low for the haemoglobin concentration in these individuals.

Material and methods

Patients

Four groups, each of 10 subjects, were studied. Group 1 comprised newly diagnosed non-pregnant

individuals with PTB, haemoglobin below 110 g/L, and having no apparent dissemination or other associated systemic illnesses. Group 2 were age- and sex-matched PTB patients with haemoglobin levels greater than 110 g/L. Group 3 consisted of otherwise healthy people with demonstrated absolute iron deficiency anaemia due to menorrhagia (n = 3), gastrointestinal tract bleeding not caused by an underlying malignancy (n = 4), or to excessive blood donations (n = 3). The haemoglobins in this group corresponded to those in group 1. Group 4 consisted of 10 healthy non-anaemic volunteers.

The study was carried out according to the requirements of the Declaration of Helsinki and with the approval of the Ethics Committee of the University of Cape Town Medical School.

Laboratory studies

These included full blood and differential count (19), biochemical profile (SMAC II autoanalyser, Technicon, Tarrytown, USA) and C-reactive protein (CRP), estimated quantitatively by immunodiffusion (20). Serum and red blood cell folate and serum vitamin B12 levels (Radioimmunoassay, Amersham International), serum iron levels, total iron binding capacity of the blood (21) and serum ferritin were determined according to standard methods. Serum Epo levels were determined by established radioimmunoassay (Incstar EPO-TRAC RIA, Minnesota, USA).

Cell cultures

Blood samples from PTB patients and iron deficient controls were diluted L/L (vol/vol) in calcium-free Hanks' buffered saline solution (HBSS), supplemented with 0.08% EDTA, and the mononuclear cells were isolated by density centrifugation on Lymphoprep (Highveld Biologicals, South Africa). Adherent mononuclear cells were cultured for 48 hours (13) and the cell-free supernatant fractions (SNF) were frozen at −70°C until analysed for TNFα by immunoradiometric assay (Amersham IRMA kit); no deterioration in activity occurs under these conditions.

HepG2 cells that had been evaluated and standardised (Department of Medical Biochemistry, Medical School, University of Cape Town; obtained through American Type Culture Collection) were plated on 24-well polystyrol dishes (Linbro Brand, Flow Laboratories, McLean, Virginia, USA) and cultured in modified Eagles' medium (MEM) with 10% foetal calf serum (Highveld Biologicals, South Africa). The cells were incubated at 37°C in humidified air, with 5% CO_2 confluent. The media were then removed and various dilutions of monocyte SNF from patients and controls were added to the cells. The HepG2 cells were incubated for a further 24 hours, after which the SNF was removed and stored at −70°C until analysis of the Epo. The cells were washed with phosphate-buffered saline and lysed with SDS-NaOH (sodium dodecyl sulphate 5 g/L in NaOH 0.1 mol/L). Total cellular protein was determined by the automated Bio-Rad dye binding microassay system (22). Samples were diluted 50-fold prior to assay in order to minimise any interference by SDS-NaOH. Epo levels were then expressed as mU/mg cellular protein.

Dose-response studies were carried out in the presence and absence of specific polyclonal goat IgG-(H34) and monoclonal IgG (101-4) TNFα antibodies (National Biological Standards Board, Hertfordshire, UK) at standardised dilutions.

Data analysis

The Mann-Whitney U-test was used to compare haemoglobin, Epo and CRP levels between the various groups. Analysis of variance (ANOVA) was used for comparison of TNFα produced by monocytes and for the levels of Epo produced by HepG2 cells in the presence of monocyte SNF from PTB patients and controls. The results are reported as mean (SEM).

Results

For matching degrees of anaemia the plasma Epo was significantly lower in group 1 than in group 3 ($p < 0.01$) (Fig. 1).

Adherent PBMC purified from anaemic PTB patients produced more TNFα (770 [173] pg/mL) than

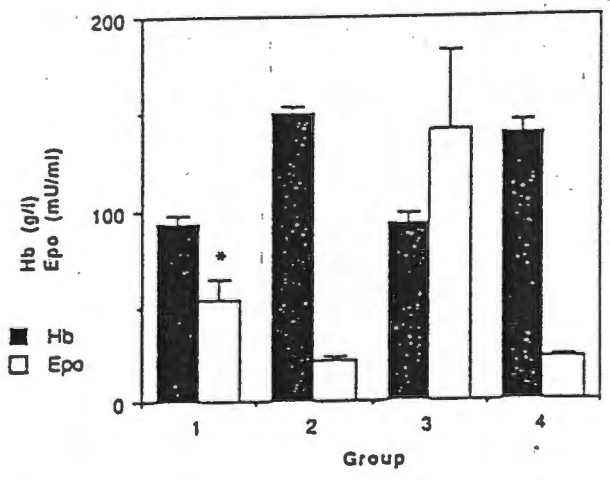

Fig. 1. Comparison of haemoglobin and erythropoietin serum levels (mean ± SEM) in anaemic PTB patients (group 1), non-anaemic PTB patients (group 2), iron deficient controls (group 3) and healthy volunteers (group 4) (*$p < 0.01$ comparing groups 1 and 3).

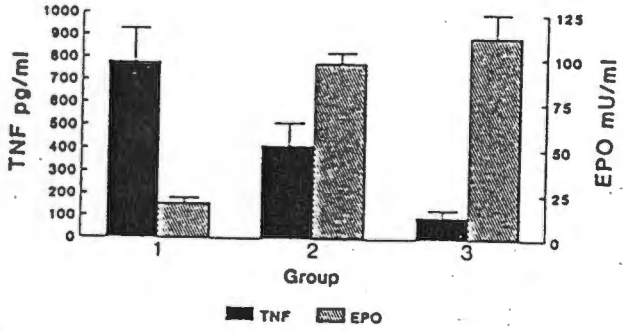

Fig. 2. Production of TNFα by PBMC obtained from untreated PTB patients with haemoglobin below 110 g/L (1), or above this level (2) and iron deficient controls (3). Also shown are the effects on erythropoietin production by HepG2 cells in the presence of monocyte SNF from the same three groups. Data represent mean (SEM). (*$p < 0.001$ comparing 1 and 3).

these cells derived from the non-anaemic patients (431 [90] pg/mL ($p < 0.001$)). The levels of this cytokine were higher in the monocyte SNF from the latter group when compared to Group 3 (98 [17] pg/mL) ($p < 0.005$) (Fig. 2). The production of Epo by HepG2 cells was markedly inhibited in the presence of monocyte SNF from patients in group 1 compared to both groups 2 and 3 ($p < 0.001$) (Fig. 2). The results shown are of experiments performed in duplicate for the individuals in each of the three groups compared with matched controls.

The effects of monoclonal and polyclonal anti-TNFα antibodies on Epo production by HepG2 cells in the presence of various dilutions of monocyte SNF were examined in the anaemic PTB group (Fig. 3). The addition of increasing concentrations of monocyte SNF resulted in a progressive reduction of Epo produced by the HepG2 cells. In the latter, the release of the hormone was elevated at the lower concentrations of monocyte SNF when the cells were incubated in the presence of optimal saturating concentrations of anti-TNFα antibodies. The fall-off reflects the use of a fixed dose of antibodies, with decline at 40% SNF attributable to increasing availability of the inhibitory cytokine.

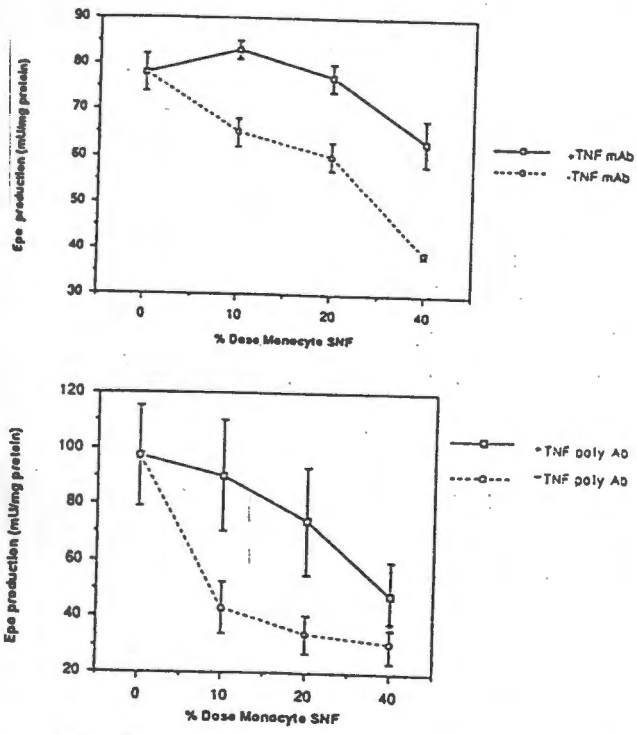

Fig. 3. The effect of specific monoclonal (mAb) (Figure 3a) or polyclonal (pAb) (Figure 3b) anti-TNFα antibody on erythropoietin (Epo) production by HepG2 cells in the presence of increasing concentrations of monocyte SNF from anaemic PTB patients. Data represent mean (SEM).

Discussion

The anaemia associated with PTB appears to be explicable, at least in part, by reference to inappropriately depressed plasma Epo levels, causally linked to the inflammatory process that leads to the release of a number of inhibitory cytokines. We found that *in vitro* production of TNFα by PBMC purified from these patients was significantly greater than in those with higher haemoglobin levels and the controls. Furthermore, monocyte SNF from this group inhibited the production of Epo by HepG2 cells *in vitro*. The suppressed release of the hormone was more pronounced than in the non-anaemic PTB patients and those with iron deficiency, in keeping with the higher levels of TNFα in the anaemic TB patients. Although the levels of this inhibitor in the non-anaemic PTB group were greater than in controls, no suppressive effect on Epo was observed. It appears, therefore, that a critical level of the cytokine may be necessary in order to block the increased requirement for Epo synthesis, thus contributing to the anaemia.

In certain patients suffering from rheumatoid arthritis or cancer, administration of recombinant human Epo has resulted in improvement of anaemia (23, 24). It appears that pharmacological doses of the hormone act directly on the marrow to stimulate erythropoiesis despite ongoing suppressant effects of inflammation and elevated circulating cytokine levels (25).

The abrogation of the suppression of HepG2 Epo released by monocyte SNF by both poly- and monoclonal anti-TNF antibodies implicates this specific cytokine in impairing Epo release *in vitro*. This is consistent with the findings of others (18), that the recombinant molecule may block the production of Epo by HepG2 cells *in vitro*. An effect of this, and possibly of other cytokines, on erythropoiesis may explain the impaired response to anaemia in patients

with PTB or other chronic inflammatory conditions (26).

In the HepG2 cell line and in normal human liver and kidney, the production of Epo is stimulated by cobalt and hypoxia; control is primarily achieved through modulation of mRNA levels (27). The mechanisms by which TNFα suppresses EPO release is the focus of further study.

In conclusion, we propose that in anaemic PTB patients the blunted EPO response may be explained by the inhibitory effects of TNFα release by monocyte-macrophages during the course of the inflammatory process.

Acknowledgements

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Characterisation of functional domains within the mouse erythropoietin 3′ enhancer conveying oxygen-regulated responses in different cell lines

Christopher W. Pugh, Benjamin L. Ebert, Osman Ebrahim, Peter J. Ratcliffe *

Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

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Abstract

We have analysed sequences within the mouse erythropoietin enhancer which are required for oxygen regulated operation in the erythropoietin producing cell line, HepG2, and in two non-erythropoietin producing cell lines; the lung fibroblastoid cell line a23, and mouse erythroleukaemia (MEL) cells. At least three critical sites were demonstrated within a 96 nucleotide sequence. Oxygen regulated operation was dependent on sites within the first 26 nucleotides. Sequences lying 3′ to this region modulated enhancer function but did not themselves convey oxygen regulated operation. In HepG2 cells these 3′ sequences co-operated to permit operation of the inducible element at a distance from a promoter, but in MEL cells 3′ sequences repressed activity of the inducible element. Though operation of this 3′ sequence differed according to the cell type, oxygen regulated operation was dependent on the same two critical sites in the 5′ region in both erythropoietin producing and non-erythropoietin producing cells. These findings support the existence of a widespread oxygen sensing system in mammalian cells which is similar to that operating in specific cells to regulate erythropoietin production, and they indicate that the system activates factors with similar DNA sequence specificity in different cells.

Key words: Oxygen; Gene; Regulation; Erythropoietin; Enhancer

1. Introduction

Erythropoietin plays a central role in the feedback regulation of erythropoiesis in response to blood loss or reduced blood oxygenation (reviewed in Refs. 1,2). Erythropoietin synthesis is almost entirely confined to restricted populations of cells within kidney and liver [3–5] where increased production is stimulated by reduced tissue oxygen delivery by a mechanism which involves increased gene transcription [6,7]. Interest has therefore focussed on transcriptional control of the erythropoietin gene as a well-defined example of oxygen regulated gene expression.

The human hepatoma cell lines Hep3B and HepG2 have provided an appropriate model for studies of this system [8]. As with physiological induction of erythropoietin production in the whole organism, erythropoietin production by HepG2 and Hep3B in tissue culture is induced by hypoxia or cobaltous ions, but not by other stresses such as cyanide exposure or heat shock [8,9]. Transfection studies in these hepatoma cells have defined an oxygen-regulated enhancer commencing approx. 120 bp 3′ to the poly(A) addition site of the human and mouse erythropoietin genes [10–14]. The active enhancer sequence lies in a region of at least 150 bp of striking homology between human and murine erythropoietin gene loci and deletional analysis of the murine enhancer showed that approx. 60–70 bp were required for enhancer action when the sequence was placed 1.4 kb 5′ to an α_1 globin reporter gene [12].

In further studies, we recently demonstrated that oxygen-regulated activity of a 96 nucleotide sequence from the mouse erythropoietin enhancer locus is not confined to the erythropoietin producing Hep3B and HepG2 cells [15]. Hypoxia inducible operation of this sequence was demonstrated in a wide variety of mammalian cell types which are not derived from liver or kidney and which do not produce erythropoietin. More detailed physiological analysis in one of these cell lines, the Chinese hamster lung fibroblastoid line, a23 [16],

* Corresponding author. Fax: +44 865 222500.

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demonstrated that the features of erythropoietin regulation observed in hepatoma cells were reflected precisely in the operation of the transfected enhancer on a heterologous promoter [15]. Thus, enhancer action was induced by exposure to cobaltous ions as well as hypoxia, but not by cyanide, arsenite or heat shock. Induction was abrogated by cycloheximide. The implication of these findings is that most mammalian cells possess an oxygen sensing and signal transduction system which is similar or identical to that which controls erythropoietin production in hepatoma cells, and which can interact with sequences within the mouse erythropoietin enhancer.

Precise definition of the sequences involved in that interaction is clearly of importance in further analysis of the system. To this end we have performed more detailed studies of the mouse erythropoietin enhancer using transfection of deleted, mutated, and re-iterated sequences, and DNAse 1 protection analysis, to define the action of subsequences within the enhancer. Since the operation of an enhancer in different cell types may result from different DNA-protein interactions [17,18] we have performed these studies in different cell types; HepG2 cells, which produce erythropoietin and support regulated operation of the mouse erythropoietin 3′ enhancer, a23 cells which support regulated enhancer function but do not produce erythropoietin, and mouse erythroleukaemia (MEL) cells which previously [15] were the only cell type not to support oxygen-regulated activity when using the 96 base pair enhancer sequence.

Inducible activity in both erythropoietin producing and non-erythropoietin producing cell types was conveyed by similar sequences and was dependent on two critical regions within the first 26 nucleotides of the mouse erythropoietin enhancer. When placed close to heterologous promoters and when re-iterated, this 26 nucleotide sequence was sufficient to convey inducible expression of reporter genes in all three cell types. Sequences lying 3′ to this region could not operate in isolation but could modulate the inducible response conveyed by the 5′ sequences; both positive and negative effects were observed and differed according to the cell type. In HepG2 cells, 3′ subsequences co-operated to permit operation of the enhancer at a distance from a promoter, but in MEL cells 3′ subsequences suppressed activity of the 5′ subsequence.

2. Materials and methods

Cell lines and culture conditions

HepG2 cells were grown in minimal essential medium with Earle's salts supplemented with 10% foetal calf serum, glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulphate (50 μg/ml). a23 cells [16] were grown in the same medium with the addition of sodium pyruvate (1 mM). MEL cells (line 585 [19]) were grown in RPMI 1640 supplemented with 15% foetal calf serum, glutamate and antibiotics as above.

Plasmid DNA

After growth in *Escherichia coli*, plasmid DNA was prepared by alkaline lysis and purified on a cesium gradient. Constructs containing deletions of the mouse erythropoietin enhancer linked to a 2570 bp *Bgl*II-*Ppu*MI fragment containing the human α_1 globin gene with 1.4 kb of 5′ flanking sequence were made in pBluescript SKII (Stratagene, Cambridge) as described previously [12] (pBSα^-). To assess the effects of distance of the enhancer sequence from the promoter, the 2570 bp α_1 globin fragment was exchanged for an otherwise identical fragment which was deleted to position −124 in the α_1 globin 5′ flanking sequence (pBS$\Delta\alpha$). The enhancer nucleotides present in these plasmids were designated by numbers related to the first nucleotide of the *Apa*I site at the 5′ end of the enhancer (Fig. 1).

Consecutive four base pair mutations of the erythropoietin enhancer, in which purines were substituted for their non-complementary pyrimidine and vice versa were made using the pALTER based Altered Sites in vitro mutagenesis system as directed by the manufacturers (Promega, Southampton). For these constructions a 460 bp sequence containing the mouse erythropoietin enhancer was first ligated into pALTER. Mutagenic oligonucleotides contained the four mutated nucleotides flanked by 14 complementary nucleotides on each side. After retrieval of the mutant plasmid and confirmation of the mutation by dideoxy sequencing, a 153 bp *Bgl*II-*Pvu*II fragment containing the mutated erythropoietin enhancer region was subcloned into polylinker sites in pBluescript SK II which contained the 2570 bp α_1 globin fragment described above (pBSα^-). A similar plasmid containing the unmutated 153 bp *Bgl*II-*Pvu*II fragment from the erythropoietin enhancer locus was made to enable a direct comparison of function between the wild type and mutated enhancer sequences.

Further subsequences from within the erythropoietin enhancer were derived either by PCR amplification or by annealing synthesised oligonucleotides. Generation of concatamers and recombination of subsequences was facilitated by using oligonucleotides in which compatible overhanging ends could be generated from *Xba*I and *Spe*I restriction enzyme sites. These sequences were inserted adjacent to the SV40 promoter in pSVGH. pSVGH contained a 198 bp *Nsi*I *Hin*dIII fragment containing the SV40 early promoter sequence linked to human growth hormone (*Bam*HI *Nsi*I fragment) in pGEM7 (Promega, Southampton)

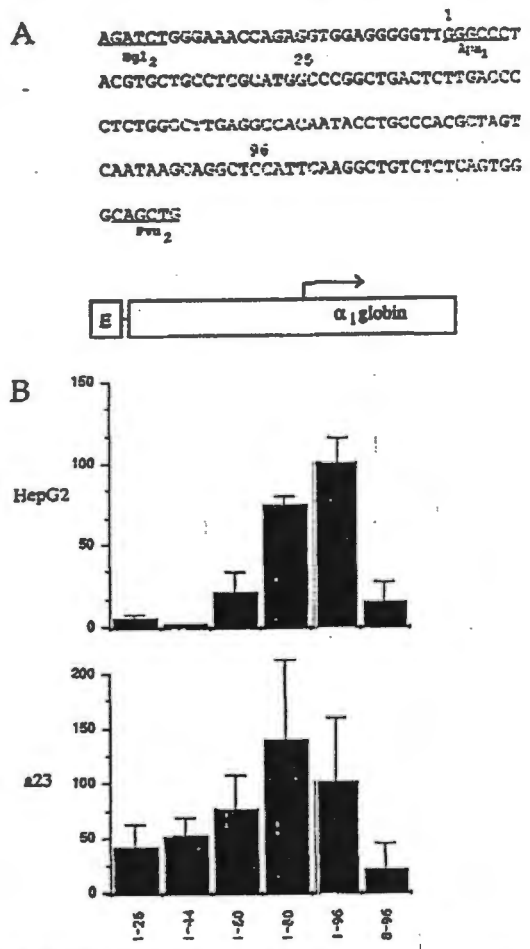

Fig. 1. Deletional analysis of the mouse erythropoietin enhancer. (A) DNA sequence of the enhancer (EMBL accession number X73471) Nucleotides are numbered from the first base of the *Apa*I site. Test plasmids used in these experiments were based on the vector pBluescript SK 11. Sequence derived from the enhancer (E) is 1.4 kb from the α_1 globin promoter (arrow). (B) Effects of deletions on enhancer function in HepG2 cells and a23 cells. Results for HepG2 include some data derived from Pugh et al. [12]. To permit direct comparison of function, plasmids containing the truncated enhancer sequences specified, no enhancer and the full length enhancer were transfected in parallel into aliquots of cells from the same pool. Transfected cells were divided for parallel normoxic and hypoxic incubation. In each transfection, expression of the test plasmid was related to a co-transfected control plasmid (FGH). The results shown are the mean ± S.D. of at least three independent transfections. No enhancer activity was observed in normoxic cells. The degree of hypoxic induction conferred by truncated sequences from the enhancer is related to that conferred by the full length enhancer in each set of experiments. In both HepG2 cells and a23 cells deletion of the nucleotides 1–8 severely reduced enhancer function. More gradual loss of function was observed with deletions at the 3′ end, where the effect of deletions differed between the cell types.

Plasmids containing no erythropoietin sequence (pSVGH) or the 168 bp *Kpn*I-*Nsi*I fragment containing the SV40 enhancer instead of erythropoietin sequence (pSVe SVGH) were used to provide a comparison with the activity of erythropoietin subsequences.

A plasmid containing 290 bp of the promoter of the mouse ferritin heavy subunit gene fused to the human growth hormone gene (pFGH) was used to control for transfection efficiency in experiments where the test plasmid contained the α globin reporter gene. In experiments where the test plasmid contained the growth hormone reporter gene, the α globin containing plasmid pBSα^- was used to control for transfection efficiency.

Transfection and incubation conditions

Cells were transfected by electroporation using a 1 mF capacitor array charged at 375 V for HepG2 cells, 400 V for a23 cells, and 425 V for MEL cells. For each transfection, approx. 10^7 cells were mixed with control and test plasmid DNA in 1 ml of RPMI 1640. α_1 globin plasmids were used at 50 μg/ml, pSVGH plasmids were used at 25 μg/ml and pFGH was used at 10 μg/ml. After transfection the cell suspension was divided for parallel 16 h normoxic and hypoxic incubations. Normoxic incubation was in humidified air with 5% CO_2. Hypoxic incubation, commencing 1 h after electroporation was in 1% O_2 with 5% CO_2 and 94% N_2 in a Napco 7100 incubator.

RNA analysis

RNA was prepared using a modified acid/guanidinium thiocyanate/phenol/chloroform extraction method (RNAzol B, Biogenesis, Bournemouth). Extracted RNA was assayed by RNAse protection. Continuously labelled antisense riboprobes were generated from α_1 globin and growth hormone templates using the SP6 system. The growth hormone riboprobe crossed the boundaries of exon 3 and protected the entire 117 nucleotide sequence of that exon. The α_1 globin riboprobe crossed the cap site of the gene; different probes, which protected 97 or 132 nucleotides of exon 1, respectively, were used when the α_1 globin reporter gene was in the test plasmid, or the control plasmid.

In all assays 3–20 μg of total RNA was subject to double hybridisation with riboprobes for α_1 globin and growth hormone. Protected fragments were separated by PAGE and quantified by scintillation counting of excised portions of the dried gel using an LKB flat-bed scintillation counter (Pharmacia-Wallac, Turku, Finland).

Each data point reported is the mean result of at least three independent transfection experiments.

Nuclear extract preparation

Nuclear extracts were prepared using a protocol derived from those of Dignam [20], and Kamakaka [21]. In outline, after parallel normoxic and hypoxic incubations, (1–5) · 10^7 cells were harvested using EDTA and resuspended in a hypotonic buffer (10 mM Na-Hepes, 5 mM Mg Cl_2, 10 mM KCl, 0.5 mM DTT, 0.1 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride

(PMSF)). Cells were then lysed by brief ultrasonication (10 Watts for 0.5 s, Soniprobe, Lucas Dawe Ultrasonics, London), such that > 85% took up Trypan blue. The nuclear pellet was mixed with an equal volume of extraction buffer (27 mM Na-Hepes, 10 mM $MgCl_2$, 300 mM KCl, 3.2 mM DTT, 0.1 mM EDTA, 1 mM benzamidine, 0.2 mM PMSF, 100 mM NaF, 1.33 μg/ml aprotinin, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin, 0.2 μg/ml bestatin and 16% glycerol) and agitated gently for 20 min at 4°C. The mix was ultracentrifuged at 100 000 × g for 30 min, the pellet and upper lipid layer were discarded and the clear supernatant, normally containing 6–10 μg/μl of protein, used directly as a nuclear extract or stored in liquid nitrogen.

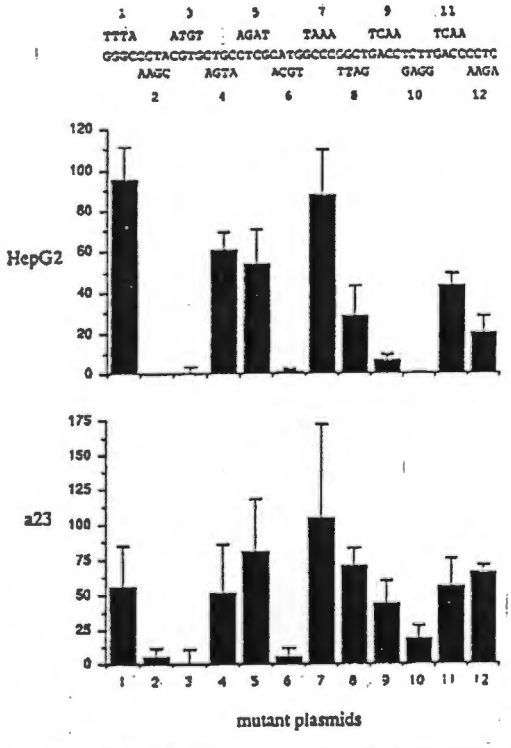

Fig. 2. Mutations define three critical regions in nucleotides 1–48 of the mouse erythropoietin enhancer. Mutations were made in a 153 bp *Bgl*II-*Pvu*II fragment from the mouse erythropoietin enhancer locus which was placed 1.4 kb from the α_1 globin promoter (see Fig. 1). Nucleotide substitutions are indicated. Plasmids containing the enhancer mutations, no enhancer and the unmutated enhancer were transfected in parallel into aliquots of cells from the same pool. Transfected cells were divided for parallel normoxic and hypoxic incubation. No enhancer activity was observed in normoxic cells. Hypoxia inducible activity of the mutant enhancers is expressed as a percentage of that of the unmutated enhancer. Data shown are the mean ± S.D. of three independent experiments. Mutations have similar effects on function in HepG2 cells and a23 cells. In each cell type mutations of nucleotide 5–12, 21–24, and 33–44 caused severe reduction, or complete ablation, of function. In HepG2 cells inducible activity of the unmutated enhancer was greater than in a23 cells, values varying from 8–17 and 3–5, respectively.

DNaseI protection assays

Restriction fragments containing the erythropoiet enhancer region were selected such that either stran could be end-labelled by DNA polymerase I (Kleno fragment) in the presence of [α-^{32}P]dCTP and used a probes. Probe ($1.5 \cdot 10^4$ cpm), approx. 50 mg of nuclea extract, and 0.5 μg of poly(dI/dC) were incubated o ice for 15 min in 6% glycerol. The final concentration of reagents in the reaction were 25 mM NaHepes, 1. mM EDTA, 3.5 mM $MgCl_2$, 30 mM KCl, 10 mM NaI 0.7 mM DTT, 0.25 mM PMSF, 5 mM benzamidine, 1 mM glycerophosphate, 2 mM levamisole, 0.1 μg/m aprotinin, 0.1 μg/ml bestatin, 0.1 μg/ml leupeptin and 0.1 μg/ml pepstatin. Titrations of DNaseI (typ IV from bovine pancreas, Sigma) diluted in 10 mM Tri (pH 8.0), were then added with $CaCl_2$ (2 mM fina concentration). After a 2 min incubation on ice, 500 μ of stop solution (0.11% SDS, 5.5 mM EDTA, and 33 mM NaCl) was added. Samples were extracted wit phenol/chloroform and chloroform, precipitated i ethanol, and washed with 80% ethanol. Pellets wer redissolved in 80% formamide running buffer and sep arated by denaturing PAGE prior to autoradiography

3. Results

Deletional analysis of the enhancer in different cell line

In previous experiments [12] we used construct which placed the mouse erythropoietin 3′ enhancer 1. kb from the promoter of an α_1 globin reporter gene t determine the minimal enhancer sequence by dele tional analysis. These experiments in HepG2 cell demonstrated that 60–70 bp were required for ful enhancer activity. As a first step in analysis of se quences required for enhancer function in differen cell lines, a similar deletional analysis was performe in a23 cells. Results are shown in Fig. 1B. As with the HepG2 cells, active sequence was sharply delineate close to the *Apa*I site at the 5′ end of the enhancer but at the 3′ end the boundary of active sequence wa less clear. In contrast with HepG2 the sequence 1–8 appeared to be more active than sequence 1–96 in a2 cells, and a greater proportion of the full length en hancer activity was retained in the shorter 5′ se quences.

Mutational analysis of the enhancer region

To identify critical regions within the enhancer, an to compare these sites in different cell lines, systematic mutations were made in the 5′ region of the enhance which was necessary for function in both HepG2 an a23 cells. Thus, 12 consecutive four base pair muta tions were made in the 48 bases lying 3′ to the *Apa* site. In each plasmid a 153 bp *Bgl*II-*Pvu*II restriction fragment containing the mutated enhancer was ligate

into the polylinker sequence of pBSα^- lying 1.4 kb 5′ to the α_1 globin reporter. Plasmids containing wild type and mutated sequences were then transiently transfected into HepG2 and a23 cells. In each transfection experiment, a single pool of cells was split to permit parallel transfections with plasmids containing no enhancer, the wild type enhancer and the mutated enhancers. After transfection, the cells were split for parallel normoxic and hypoxic incubations so that it was possible to compare directly the level of induction conveyed by wild type and mutant enhancer sequence. Results are shown in Fig. 2. In these plasmids, no constitutive action of the enhancer was observed in either a23 cells or HepG2 cells. Inducible activity of the wild type enhancer was greater in HepG2 than in a23 cells (8–17-fold compared with 3–5-fold), but the effect of mutation was very similar. In each cell type, nucleotides substitutions in three regions corresponding to the mutations in plasmids 2–3, 6 and 9–11 resulted in severe reduction of enhancer function. In contrast, mutations in plasmids 4, 5 and 7 had little effect on activity in either cell type.

DNaseI protection analysis of the enhancer sequences

To analyse further the structure of the enhancer DNaseI protection assays were performed using nuclear proteins extracted from aliquots of HepG2, a23 cells, and MEL cells following parallel incubation under normoxia and hypoxia.

Fig. 3 shows the results obtained using a probe labelled 5′ to the functionally defined sequence. Using nuclear extracts from HepG2 cells striking protection of nucleotides 28 to 49 was observed. This area was also protected when nuclear extract from a23 cells was used, although the protected region extended further 3′ and covered nucleotides 28 to 66. Nuclear extracts from MEL produced a similar footprint but the protection was less complete. Addition of all nuclear extracts resulted in increased nuclease activity at nucleotide 23, when compared with the no extract lanes. Another area of protection was observed in the 5′ region of the enhancer covering nucleotides −11 to 9 relative to the *Apa*I site.

When equal concentrations of extracts from normoxically and hypoxically incubated cells of each type were compared no change in the extent or degree of DNaseI protection was seen.

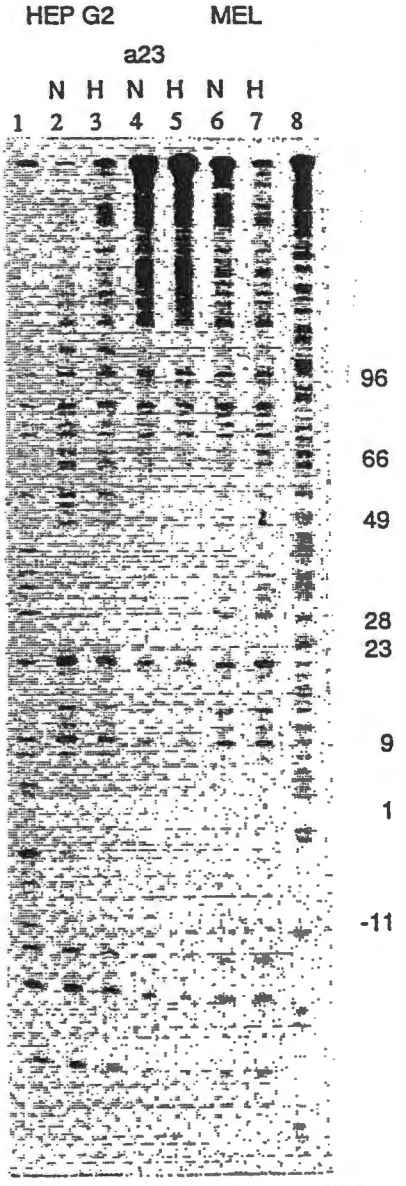

Fig. 3. DNAseI protection analysis of the mouse erythropoietin enhancer. The 5′ end-labelled probe was a 484 bp *Bgl*II-*Sph*I fragment containing the mouse erythropoietin enhancer linked to polylinker sequence from pBluescript SK11. Nuclear extract was prepared after parallel 16 h incubations of cells in normoxia, 21% O_2 (N) and hypoxia, 1% O_2 (H). Lane 1, no extract; lanes 2 and 3, 50 μg of extract from normoxic and hypoxic HepG2 cells; lanes 4 and 5, 50 μg of extract from normoxic and hypoxic a23 cells; lanes 6 and 7 70 μg of extract from normoxic and hypoxic MEL cells. Lane 8 is a Maxam and Gilbert G+A column from the same probe. The regions protected are indicated using the numbering system shown in Fig. 1.

Functional analysis of subsequences

Since subsequences within enhancers may operate in isolation when placed close to a promoter or when re-iterated, further expression studies were performed using such elements placed immediately 5′ to the SV40 promoter. First the operation of elements consisting of nucleotides 1–26 and 25–60 was compared.

Nucleotides 1–26 contain the two critical regions defined by mutants 2–3 and 6, whereas nucleotides 25–60 contain a direct repeat sequence TGAC-CTCTTGACCC resembling a steroid/thyroid receptor binding element and defined as functionally important by mutants 9–11 (see above). When expressed in HepG2 cells hypoxic expression was induced with the plasmids containing sequence 1–26 adjacent to the SV40 promoter but not with those containing sequence

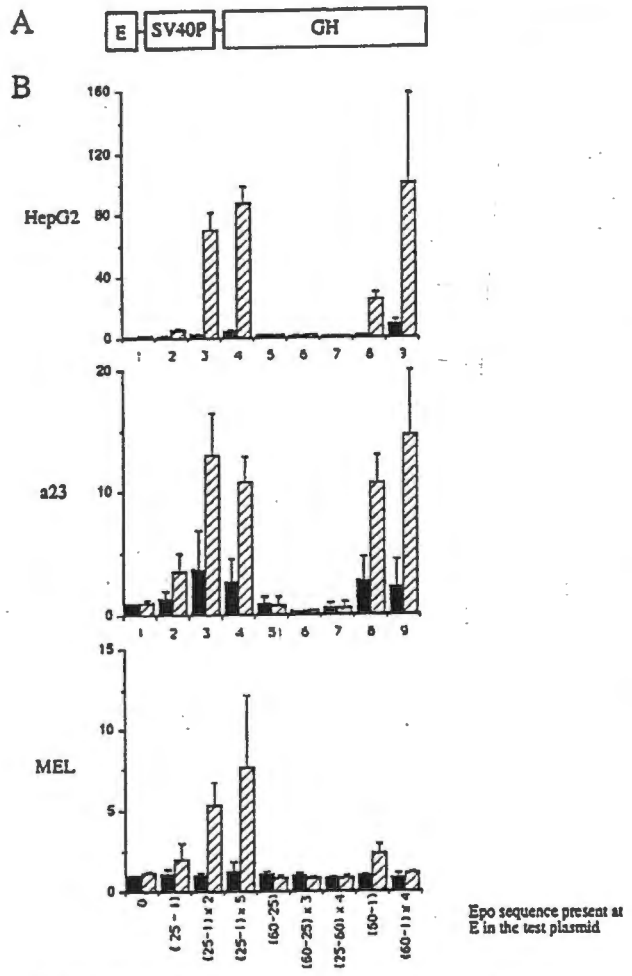

Fig. 4. Action of subsequences from the mouse erythropoietin enhancer on an adjacent SV40 promoter. The structure of the test plasmids is shown in (A), where E represents erythropoietin enhancer sequence, SV40P represents the SV40 early promoter and GH represent the body of the growth hormone gene. The erythropoietin enhancer sequence (E) used in each test plasmid is indicated beneath the corresponding bars of the histogram, numbered as in Fig. 1. A co-transfected plasmid containing the α_1 globin gene alone (pBSα^-) was used to correct for transfection efficiency. Expression is normalized to that of the enhancerless pSVGH in normoxic cells and represents the mean ± S.D. of three independent experiments (B). Inducible activity is conveyed by sequence 1–25 in all cell types. Inducible activity was much increased by concatamerization of this sequence which also conveyed some constitutive activity in normoxic cells $(1–25)_2$ and $(1–25)_5$. In contrast, neither constitutive nor inducible activity was conveyed by sequence (25–60) in any cell type. Concatamers of this sequence were tested in either orientation $(25–60)_3$ and $(60–25)_4$ but none had any action. The monomeric sequence, 1–60, was more active than the monomeric sequence, 1–25 in HepG2 cells and a23 but not MEL cells.

25–60. This difference was most marked when concatamers of these sequences were compared. Re-iteration of sequence 1–26 resulted in large increases in both normoxic expression and in the amplitude of hypoxic induction, with expression in hypoxic HepG2 exceeding that of the control plasmid containing the SV40 promoter alone by a factor approaching 100-fold. In contrast, re-iterations of sequence 25–60 produce no constitutive increase in expression in normox HepG2 cells and no inducible expression in hypox HepG2 cells (Fig. 4B).

When these plasmids were expressed in a23 cell the pattern of expression was very similar. Activi resided in the sequence 1–26 but not in the sequenc 25–60, although the level of induction was not as gre as that observed in HepG2 cells (Fig. 4B). Interes ingly, when the constructs were expressed in MEL cel inducible activity was also observed. Again, activity wa conveyed by the sequence 1–26 but not sequence 25–6 (Fig. 4B).

Also of interest is comparison of the action of se quence 1–60 with sequence 1–26 when placed adjacer to the SV40 promoter. In HepG2 cells and a23 cell there was an increase in the inducible activity of se quence 1–60 when compared with sequence 1–26. Th effect was not observed in MEL cells (Fig. 4).

Since mutational analysis had indicated the exis tence of two critical regions within the sequence 1–2 short sequences including each of these sites wer inserted either as monomers or multimers adjacent t the SV40 promoter and expressed in HepG2 cells. Th two sequences 4–15 and 16–27 were also rejoine using an 8 bp spacer (4–15) TCTAGTCC (16–27. None of these sequences conveyed activity when ex pressed in HepG2 cells (Table 1).

Effect of distance from a promoter

While both deletional and mutational analysis i Hep G2 cells indicated that sequences 3′ to nucleotid 26 were obligatory for enhancer function when th sequence was placed 1.4 kb from the α_1 globin pro moter, these sequences were not obligatory and had n activity in isolation when placed adjacent to an SV4 promoter. To distinguish whether this difference was function of distance from the promoter or some spe cific aspect of promoter sequence, expression of plas mids containing subsequence 1–25 or subsequenc 1–96 approx. 0.1 and 1.4 kb from the α_1 globin pro moter was compared. In HepG2 cells, sequence 1–9 was almost equally active in both positions wherea subsequence 1–25 was only active close to the pro

Table 1

Construct	Constitutive	Hypoxic/ Normoxic
Control	1.00	1.46
(4–15)	1.04	1.41
(4–15)×2	0.69	1.40
(4–15)×4	0.76	1.31
(4–15)(15–4)	0.76	1.39
(15–4)×2	0.92	1.24
(27–16)×2	0.87	0.91
(16–27)×5	0.82	1.49
(4–15)N_8(16–27)	1.20	0.92

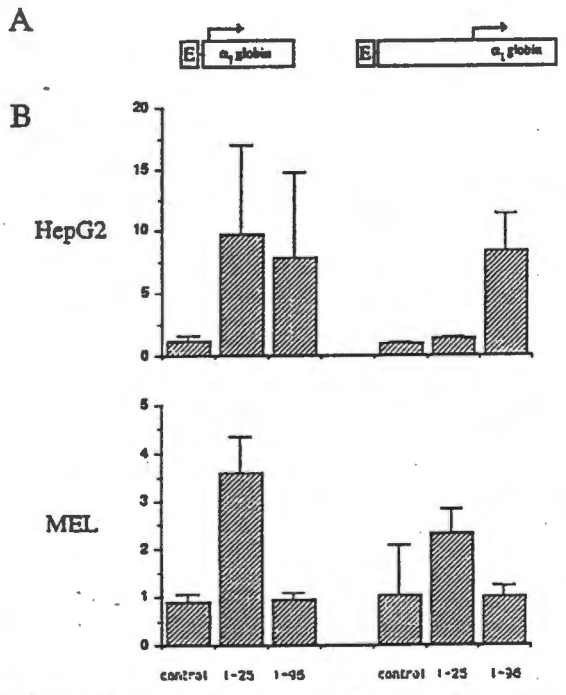

Fig. 5. Effect of distance from a promoter on function of the mouse erythropoietin enhancer. The test plasmids which were used in these experiments are shown (A). The pairs of plasmids were identical except that one contained 124 bp of 5′ flanking sequence from the α_1 globin gene and the other contained 1.4 kb of this sequence separating the enhancer from the α_1 globin promoter. A plasmid (FGH) was co-transfected to control for non-specific variation in transfection, RNA synthesis and RNA recovery. Transfected cells were divided for parallel normoxic and hypoxic incubation. Normoxic and hypoxic expression of the test plasmid were normalised with respect to expression of the control plasmid. The values plotted represent the ratio of normalised hypoxic to normoxic expression from three separate transfections (mean ± S.D.). In HepG2 cells nucleotides 25–96 were necessary for enhancer function at a distance from the α_1 globin promoter but not when the enhancer was close to the promoter. In MEL cells nucleotides 1–96 had no constitutive or inducible activity when placed either near to or distant from the α_1 globin promoter whereas inducible enhancer activity was observed with nucleotides 1–25, indicating that in this cell line nucleotides 25–96 could suppress activity of the inducible element.

moter (Fig. 5A). In MEL cells subsequence 1–25 was also active close to the promoter and retained a little activity at a distance. In contrast, sequence 1–96 was inactive in MEL cells whether it was near to or distant from the α_1 globin promoter (Fig. 5B), thus indicating the potential for regions within the sequence 25–96 to repress inducible activity in these cells.

4. Discussion

In this paper we present evidence for functionally distinct regions within the mouse erythropoietin 3′ enhancer and show that similar sequences at the 5′ portion of this enhancer are required in different cell lines for interaction with a widespread oxygen sensing system.

Deletion of sequence close to the *Apa*I site at the 5′ end of the enhancer (construct 96–8, Fig. 1) sharply reduced enhancer activity indicating that these eight nucleotides contained bases critical for inducible enhancer operation. However, the length of the minimal functional enhancer indicated that enhancer function was most probably dependent on several other critical sequences. This was borne out by the mutational analysis of the first 48 nucleotides (Fig. 2) which defined three critical sites, corresponding to nucleotides 5–12, 21–24 and 33–44. The sequences represented by nucleotides 5–12 and 21–24 do not have homology with known transcription factor binding motifs. Mutations affecting nucleotides 33–44 disrupt the sequence TGACCTCTTGACCC which it has been suggested may represent a binding site for an orphan receptor of the steroid/thyroid hormone family [14].

The constructs used in these experiments contained the enhancer element separated from the α_1 globin promoter by 1.4 kb. All three regions within the enhancer were absolutely necessary for function in this setting. These sites might each convey inducible interactions; alternatively some might simply play a permissive role for enhancer function. Isolated sequences within enhancers which are inactive when placed at a distance from a promoter may operate when re-iterated or placed close to the promoter [18,22,23]. To test the subsequences individually for their ability to convey inducible responses we therefore placed monomers or concatamers of each element adjacent to the SV40 promoter.

Concatamers of nucleotides 1–26, which contained the 5′ critical sites, but neither of the TGACC$^{C}/_{T}$ half-sites, were compared directly with concatamers of nucleotides 25–60, containing the TGACC$^{C}/_{T}$ direct repeat. The behaviour of the two regions was quite different. Concatamers of nucleotides 1–26 were highly active and showed inducible activity which was substantially greater than even the full length enhancer. In contrast, neither monomers nor different concatamers of nucleotides 25–60 showed any activity. The independent operation of nucleotides 1–26 in the pSVGH plasmids was confirmed by the inducible operation of this sequence in pBS$\Delta\alpha$1–25, which contained a different heterologous promoter and reporter gene. The two critical subsequences within this region indicated by mutation of nucleotides 5–12 and 21–24 did not appear to function independently since a variety of concatamers of nucleotides 4–15 or 15–27 were not functional. Furthermore, introduction of spacing nucleotides TCTAGTCC in construct 4–15(N_8)16–27 ablated function. Thus, these studies define the inducible operation of a 26 nucleotide sequence containing two sites which are critical for function.

Two recent studies of the human erythropoietin enhancer have focussed on two different portions of the human enhancer, and have shown that mutations in each region can destroy enhancer function [13,14]. The region which we mutated in the mouse erythropoietin enhancer was somewhat more extensive, and covered the regions which are homologous to both of those examined in the human studies. Despite the use of a different system of nucleotide substitution, the results of the current studies are entirely consistent with the experimental observations in each of the studies of the human enhancer. The critical regions defined by mutations 2–3 and 6 correspond to sequences designated BS-1 and BS-2 in one study [13], whereas mutations 9–11 affect the directly repeated TGACC$^{C}/_{T}$ half sites which were shown to be critical for function in the other study [14]. Thus, at least three functionally critical regions have been defined in the murine and human erythropoietin enhancers. Different conclusions were reached in the two studies of the human enhancer as to the function of each region. Blanchard et al. [14] argued that the directly repeated half-sites were the critical site of interaction, and postulated that modification of a factor at this site mediated the oxygen regulated response. In contrast, Semenza and Wang [13] demonstrated an inducible DNA binding activity using an oligonucleotide from the 5′ region of the enhancer, and found that a 33 bp oligonucleotide extending to include only 4 bp of one of the directly repeated half-sites retained some inducible activity which could be restored to the level shown by the full length enhancer by re-iteration. They postulated that the inducible interaction took place with sites at the 5′ end of the enhancer with the direct repeat element functioning to amplify the response. Our results, using a more extensive set of concatamerized subsequences from the murine erythropoietin enhancer support this interpretation.

When the non-erythropoietin producing a23 cells are compared with HepG2 cells the same two critical sites were necessary for function in the 5′ region of the enhancer. Furthermore, in both HepG2 and a23 cells, sequences lying 3′ to this region were important for operation at a distance, amplified the response when the enhancer sequence was close to the promoter, but did not function independently. In our first report of inducible operation of the mouse erythropoietin enhancer in non-erythropoietin producing cells [15], we performed physiological and pharmacological studies to show that the inducible response was distinct from other cell stress responses such as heat shock, and resembled the inducible response of the native erythropoietin gene in hepatoma cells. We therefore argued that a similar or identical mechanism of oxygen sensing and signal transduction must operate widely, most probably inducing the expression of other genes in non-erythropoietin producing cells. Such a system could induce gene expression by activation of a variety of transcription factors which might vary between different cell types and recognize different motifs in cis-acting regulatory sequences [17,18]. However, the current demonstration that similar subsequences within the erythropoietin enhancer are critical for function in different cell lines strongly suggests that common factors are operating this response in different cell lines.

In the DNAse1 protection assays we did not observe inducible changes in protection of the critical sequences in the 5′ region of the enhancer. Nor was DNAse1 protection observed at similar sites in recent analyses of the human erythropoietin enhancer [13]. However, using electrophoretic mobility shift assays, Semenza and Wang have observed inducible binding of a nuclear factor from Hep3B cells, which they designated HIF-1, to nucleotides 3–21 of the human enhancer [13]. Our results are therefore consistent with the binding of such a factor to the mouse erythropoietin enhancer and strongly suggest that it operates in other cell lines. Indeed, during the preparation of this manuscript, a paper reporting HIF-1 binding activity in a variety of mammalian cell lines in which the erythropoietin gene is not transcribed has been published [24].

Using nuclear extract from HepG2 cells in DNAseI footprinting studies striking non-inducible protection of nucleotides 29 to 50, which include the TGACC$^{C}/_{T}$ repeat element, was observed. This footprint was closely similar to that described on the human enhancer using extract from Hep3B cells [13,14]. However, a larger footprint extending over a further 18 nucleotides 3′ to this region was observed in a23 and MEL. Although this indicates differences in the DNA–protein interactions with the 3′ end of the enhancer it does not fully explain the functional data. For instance, deletion analysis in HepG2 cells implicated functionally important sequences lying 3′ to nucleotide 50 which were necessary for enhancer operation at a distance, but which were not protected. It therefore appears that, in addition to the TGACC$^{C}/_{T}$ direct repeat, further subsequences in the 3′ region of the enhancer modulate the function of the inducible element. Precise definition of these sequences and their interactions will require further work.

One unexpected result was inducible operation via the 1–26 nucleotide sequence in MEL cells. These cells were the only cell type in our previous study [15] which was unable to support oxygen regulated reporter gene expression using plasmids containing the 1–96 nucleotide enhancer sequence. This discrepancy was explained when inducible expression of the α_1 globin reporter was compared in different sets of plasmids containing nucleotides 1–25 and 1–96, showing that sequences in the region 25–96 could operate repressively. Thus, although the sequences conveying in-

ducible expression in the first 25 nucleotides of the enhancer appear to operate similarly in different cells the current studies demonstrate the potential for modulatory effects to obscure the operation of the inducible element in different cells.

Although our previous studies indicated that the hypoxically inducible response was widespread in mammalian cells, failure to demonstrate inducible action in MEL suggested that the mechanism was not universal [15]. It is clear from the current studies that in fact the inducible element can operate in MEL cells. Others have previously reported the absence of inducible responses in HeLa cells [10]. In the light of the above observations we have now tested these cells too and found inducible responses using constructs containing nucleotides 1–26. We have now tested nineteen cell lines (Ref. 15, this report and data not shown) and have yet to find one which does not have the capacity to modulate oxygen dependent changes in reporter gene expression by the sequence 1–26.

In addition to the promoters used in these experiments, other transcriptionally active sequences from the SV40 enhancer, the herpes-simplex virus thymidine kinase gene promoter and the hypersensitive site 2 of the human β globin locus control region [25] do not show hypoxically inducible activity [11,12,15] (and data not shown) confirming the sequence specificity of this widespread response.

Based on responses obtained by exposure of hepatoma cells to transition elements, haem synthesis inhibitors, and haem protein ligands such as carbon monoxide, Goldberg et al. [9] proposed that the oxygen sensor controlling erythropoietin production by hepatoma cells might be a haem protein. The existence of a similar mechanism of oxygen sensing in all the mammalian cells we have tested indicates that this mechanism may well mediate oxygen-regulated expression of other genes in mammalian cells not specialized for the production of erythropoietin. It is also possible that the system is homologous with one or more of the oxygen sensing systems present in lower organisms, where the involvement of haem and haemoproteins in oxygen-regulated gene expression is well established [26–28].

In mammalian cells, the expression of several genes implicated in vascular growth is induced by hypoxia in a manner which resembles induction of erythropoietin gene expression in hepatoma cells. Platelet derived growth factor β chain and endothelin expression is induced in hypoxic human umbilical vein endothelial cells [29,30]; as with the erythropoietin gene hypoxically induced expression was severely reduced by exposure to carbon monoxide [29]. Expression of the vascular endothelial growth factor gene is induced by hypoxic exposure in a variety of tissue culture cells; induction is sensitive to cycloheximide as is the case for induction of erythropoietin expression [31]. Oxygen regulated changes in gene expression also contribute to control of cellular metabolism. Expression of the genes for several glycolytic enzymes is induced by hypoxia, whereas expression of the gene for the gluconeogenic enzyme phospho*enol*pyruvate carboxykinase is reduced by hypoxia [32–34]. This effect of hypoxia on phospho-*enol*pyruvate carboxykinase gene expression is mimicked by cobalt at doses similar to those which induce erythropoietin gene expression [34].

The definition of the *cis*-acting elements which are critical for interaction with the inducible system and the demonstration that the same sequences convey the interaction in non-erythropoietin producing cell lines should focus attempts to define and compare *cis*-acting sequence responsible for oxygen regulated expression of these other genes and to detect whether similar *trans*-acting mechanisms are indeed involved.

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