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THE BIOGENESIS OF ERYTHROPOIETIN DURING INFLAMMATION

BY

HENRY MARTIN JOHN LENG

[B.Sc., M.Pharm.(UWC), M.Sc.(Med. Sc.)(STELL.)]

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DEDICATION

TO MY MOTHER, ELIZABETH, WIFE, ZELDA, AND DAUGHTER, TARNÉ

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THE BIOGENESIS OF ERYTHROPOIETIN DURING INFLAMMATION

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ABSTRACT

Anaemia frequently accompanies chronic inflammatory diseases like rheumatoid arthritis and cancer. It is postulated to result primarily from the suppression of erythropoiesis by inflammatory cytokines. A contributing factor could be the inhibition of erythropoietin synthesis which may also be mediated by cytokines. Erythropoietin is the hormone which regulates erythropoiesis. The aims of this project were to investigate whether cytokines can indeed suppress erythropoietin production, and to determine whether the erythropoietin response in experimental models of acute and chronic inflammation was appropriate for the associated anaemia.

Macrophage-conditioned medium, interleukin-1ß, interleukin-6, tumour necrosis factor-α, and neopterin were assayed for inhibition of erythropoietin synthesis by HepG2 cells in culture. All, except neopterin, effected dose-dependent reductions in the secretion of the hormone. Interleukin-1ß and tumour necrosis factor-α down-regulated erythropoietin gene transcription, whereas interleukin-6 inhibited a post-transcriptional process.

Rats with acute inflammation developed a mild anaemia which evoked an increase in their serum levels of erythropoietin. The serum erythropoietin levels were optimal, since rats with acute inflammation and severe phenylhydrazine-induced anaemia did not have lower levels of the hormone than controls with a similar degree of anaemia, but without acute inflammation. Erythropoietin is, therefore, not an acute phase reactant.

Mice with cancer developed a progressive anaemia which was not due to bone marrow invasion by tumour cells. During the first fourteen days after inoculating them with cancer cells, the mice responded by increasing their serum levels of erythropoietin as

the anaemia worsened. The erythropoietin response was appropriate when compared to mice with the same degree of phenylhydrazine-induced anaemia. Erythropoietin levels measured in mice with tumours older than fourteen days were significantly lower than those of control mice with the same degree of experimental anaemia. These animals were very cachectic, suggesting that a blunted erythropoietin response may depend on disease activity.

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LIST OF ABBREVIATIONS

Aa	amino acid
*Acc*I	restriction enzyme GT↓(A/C)(G/T)AC
ACD	anaemia of chronic disorders
ACTH	adrenocorticotrophic hormone
AIA	adjuvant-induced arthritis
AIDS	acquired immunodeficiency syndrome
AMP	adenosine-5'-monophosphate
APR	acute phase response
ATP	adenosine-5'-triphosphate
BFU-E	burst-forming unit-erythroid
*Bgl*II	restriction enzyme A↓GATCT
bp	base pair
C-terminal	carboxyl amino acid terminal
cAMP	cyclic AMP
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CFU-E	colony-forming unit-erythroid
CIA	collagen-induced arthritis
CRP	C-reactive protein
CTP	cytidine-5'-triphosphate
CV	coefficient of variation
dATP	deoxyATP
dCTP	deoxyCTP
DEPC	diethyl pyrocarbonate
dGTP	deoxyGTP
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxyTTP
E. coli	*Escherichia coli*
*Eco*RI	restriction enzyme G↓AATTC
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay

Epo	erythropoietin
EtBr	ethidium bromide
FCS	foetal calf serum
fl	femtolitres
FVA	Friend virus that produces anaemia
g	gram
GM-CSF	granulocyte-macrophage colony stimulating factor
GTP	guanosine-5′-triphosphate
h	hour
Hb	haemoglobin
Hct	haematocrit
Hepes	N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
*Hin*dIII	restriction enzyme A↓AGCTT
HIV	human immunodeficiency virus
IC_{50}	half maximal inhibitory concentration
IL-1	interleukin-1
IL-6	interleukin-6
iu	international units
kbp	kilobase pair
kd	dissociation constant
kD	kilodalton
LB	Luria-Bertani medium
LE	Long-Evans rats
LPS	lipopolysaccharide
M	molar
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCM	macrophage-conditioned medium
MEM	minimum essential medium
mg	milligram
min	minutes
MK	megakaryocytes
ml	millilitres
mM	millimolar

MOPS	3-morpholinopropanesulfonic acid
mRNA	messenger RNA
MTT	3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide
N-terminal	amino-amino acid terminal
ng	nanogram
nm	nanometers
OD	optical density
oligo(dT)	oligodeoxythymidylic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PKC	protein kinase C
poly(A)$^+$RNA	polyadenylated mRNA
*Pst*I	restriction enzyme CTGCA↓G
PZN	phenylhydrazine
RA	rheumatoid arthritis
RCC	red cell count
rhu-Epo	recombinant human Epo
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	ribonuclease inhibitor
rpm	revolutions per minute
rRNA	ribosomal RNA
s	seconds
SAA	serum amyloid A
SAP	serum amyloid P
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLS	sodium lauryl sulphate
SSC	standard saline citrate
TBE	tris-borate-EDTA
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine

TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
u	units
UTP	uridine-5'-triphosphate
uv	ultraviolet
w/v	weight per volume
WCC	white cell count
*Xba*I	restriction enzyme T↓CTAGA
α	alpha
β	beta
μ	micro
°C	degrees Celsius
%	percent

CHAPTER ONE

ERYTHROPOIETIN: BIOCHEMISTRY, PHYSIOLOGY, AND CLINICAL APPLICATION.

1.1 INTRODUCTION

In the middle of the last century, Dennis Jourdanet observed that Mexicans living at high altitudes had an increased number of red blood cells in their circulation. He and a friend, Paul Bert, realised that such an increase could provide survival advantages for people living at a low oxygen tension (quoted in Erslev, 1990). In 1906, Carnot and DèFlandre suggested that this response was mediated by a humoral factor (quoted in Sherwood, 1984). Definitive evidence that erythropoiesis is controlled by a circulating factor, acting directly on the marrow, was provided by Reismann (1950) and Stohlman *et al.* (1954). Reismann (1950) observed, using parabiosed rats, that lowering the blood oxygen saturation to 63% in one of the rats was followed by increased erythropoiesis in both the hypoxic rat and the partner, which was breathing room air and had a blood oxygen saturation of 97%. Stohlman and co-workers (1954) studied a patient with a patent ductus arteriosus and normal oxygen saturation above the diaphragm, but with decreased oxygen saturation below the diaphragm. Erythroid hyperplasia was observed in the sternal marrow, despite a normal marrow oxygen saturation, suggesting that a humoral factor produced below the diaphragm, in response to hypoxia, regulated erythropoiesis. Subsequent studies revealed that the factor is produced in response to all kinds of hypoxia and it was named erythropoietin (Epo) in recognition of its role as the hormone involved

exclusively with red cell production (Sherwood, 1984).

1.2 CHEMISTRY AND MOLECULAR BIOLOGY OF ERYTHROPOIETIN

The purification of Epo from human urine was fully accomplished (Miyake *et al.*, 1977). It was found to be a glycoprotein with a molecular mass of 34 000 daltons and containing approximately 30% carbohydrate of which 11% was sialic acid, 11% total hexose, and 8% N-acetylglucosamine. The protein sequence consisted of 166 amino acid residues with a calculated molecular mass of 18 398 daltons (Wang *et al.*, 1985; Lai *et al.*,1986).

The elucidation of the primary amino acid sequence of Epo enabled the construction of oligonucleotide probes for the isolation of human (Jacobs *et al.*, 1985; Lin *et al.*, 1985), monkey (Lin *et al.*, 1986), and murine (McDonald *et al.*, 1986; Shoemaker and Mitsock, 1986) Epo genes. In all three species, the Epo gene exists as a single copy and is highly conserved, with the monkey and murine DNA coding sequence having a 94% (Lin *et al.*, 1986) and 80% (Shoemaker and Mitsock, 1986) homology, respectively, with the human gene. The human Epo gene is located on chromosome 7 (Law *et al.*, 1986; Powell *et al.*, 1986) and, more specifically by *in situ* hybridisation, has been localized to region q11-q22 (Law *et al.*, 1986). It is contained in a 5.4 kilobase fragment of the genomic DNA and consists of four introns and five exons for a 193 amino acid peptide. The product of the gene includes a 27 amino acid signal peptide, which is cleaved during secretion to release the 166 amino acid hormonal peptide(Lin *et al.*, 1985).

Glycosylation of the hormonal peptide seems to be absolutely necessary for its *in vivo* activity (Takeuchi *et al.*, 1989). Hormonal activity was abolished after sialic acid residues were removed. The asialoerythropoietin had, however, full activity

when assayed *in vitro* (Goldwasser *et al.*, 1974). The apparent loss of activity of the asialoerythropoietin *in vivo* can be explained by its rapid clearance from the circulation. When terminal sialic residues are removed from the oligosaccharides, galactose residues become the new terminal sugars. These galactose-terminated glycoproteins may then be recognised by receptors present in hepatocytes and are internalized by endocytosis, followed by digestion with lysosomes (Morell *et al.*, 1968; Nielsen *et al.*, 1989). Thus, it is apparent that Epo should be fully glycosylated, in particular sialylated, to accomplish *in vivo* hormonal activity.

1.3 SITE OF ERYTHROPOIETIN PRODUCTION

Jacobson *et al.*(1957), demonstrated that the kidney was the major organ responsible for increased serum Epo levels. Rats with bilateral nephrectomies no longer increased Epo production in response to anaemia. Subsequent studies have provided ample support for this proposal. It was shown that the normal isolated kidney will synthesize Epo when perfused with a serum-free medium (Erslev, 1974) and that this synthesis is augmented when perfused at low pO_2 (Fisher and Langston, 1968). Serum Epo levels were also found to be extremely low in patients with end-stage renal failure and severe anaemia, but after successful renal transplantation these levels reverted to normal (Besarab *et al.*, 1987).

Although the kidney is now widely accepted as the source of Epo in the adult mammal, it is by no means the only organ involved in Epo biogenesis. Anephric rodents have been found to retain 10% of their capacity to produce Epo (Jacobson *et al.*, 1959) and anephric patients also had some Epo in their plasma (Mirand *et al.*, 1968; Naets and Wittek, 1968). Since hepatectomy abolished the residual Epo production of anephric rats (Fried, 1972), the

liver appears to be the primary site of extrarenal Epo production. Extrarenal Epo synthesis in response to anaemia was also found to correlate with liver regeneration after partial hepatectomy (Naughton *et al.*, 1977). In addition, the liver appears to be the major site of Epo production during foetal life (Zanjani *et al.*, 1977; Koury *et al.*, 1988).

The development of genetic probes for Epo mRNA has provided additional means for localizing Epo production. Initial studies established the anaemic mouse or rat as good models for studying Epo production (Bondurant and Koury, 1986). RNA, isolated from the kidney and liver of normal and anaemic mice, was hybridized with a radioactive Epo mRNA-specific probe. Trace amounts of Epo mRNA were detected in normal kidneys when polyadenylated RNA was analysed. After anaemia was induced, an increase in Epo mRNA was noted by 1.5 h with a rise of more than 200 fold by 4 h. Small amounts of Epo mRNA were also detected in the anaemic liver (Bondurant and Koury, 1986). Similar findings were reported by Schuster *et al.*(1987), who studied Epo mRNA expression in anaemic rats. Chorh *et al.* (1991), using the more sensitive RNase protection assay, detected Epo mRNA in the kidney, liver, and even the lung of uraemic rats. Kidney and plasma Epo levels measured by radioimmunoassay paralleled the changes in Epo mRNA levels, suggesting that Epo production in response to hypoxia represents *de novo* synthesis. When hypoxia was discontinued, rat kidney Epo mRNA decreased to undetectable levels by 3 h, indicating a fairly short half-life (Schuster *et al*, 1987).

When nuclei of anaemic kidneys and Hep3B cells, which secrete Epo, were isolated and examined, both were found to have a higher level of transcribed Epo mRNA after hypoxia (Schuster *et al.*, 1989; Costa-Giomi *et al.*, 1990). Cycloheximide administration before hypoxia prevented the increase in mRNA, suggesting that protein synthesis was necessary (Goldberg *et*

al., 1988; Caro *et al.*, 1989). These nuclear run-off experiments indicate that the increase in Epo mRNA can, at least partially, be attributed to an increased rate of transcription of the Epo gene.

1.4 IDENTIFICATION OF EPO-PRODUCING CELLS

The availability of labelled RNA or DNA probes for *in situ* hybridisation of Epo mRNA has provided a method for identifying the individual cells that make Epo. Two groups (Koury *et al.*, 1988; Lacombe *et al.*, 1988), have demonstrated that a peritubular interstitial cell, outside the tubular basement membrane, was the renal cell that produced Epo mRNA during hypoxia. These cells were found in the cortex or outer medulla. The cells had an endothelial-like morphology (Koury *et al.*, 1988), and they contained factor VIII-related antigen (Lacombe *et al.*, 1988). Hence, peritubular capillary endothelial cells have been considered the most likely candidates for expressing Epo mRNA in the hypoxic kidney. Nevertheless, the evidence for the peritubular production site is not accepted by all authors. Maxwell *et al.* (1990), using a ^{32}P-labelled DNA probe corresponding to part of exon IV of the murine Epo gene, reported that Epo mRNA was localized in tubular cells of the hypoxic mouse kidney. Although there is no apparent explanation for this discrepancy, it clearly demonstrates that *in situ* hybridisation findings require cautious interpretation.

When the number of interstitial cells with Epo mRNA was measured in relation to increasing anaemia, it was found that these cells increased in an exponential manner in parallel with the exponential increase in total Epo mRNA and serum Epo (Koury *et al.*, 1989). It was, therefore, proposed that when anaemia becomes more severe, additional cells are recruited that synthesize Epo, with the individual cells responding maximally

once activated (Koury *et al.*, 1989). It has been estimated that 20% to 30% of the total interstitial population of the inner cortex, but less than 10% of the interstitial cells in the subcapsular cortex, produced Epo (Koury *et al.*, 1989). Thus, Epo-producing cells may represent a specialised cell type within the cortical interstitium, rather than a generalised cell type (Krantz, 1991). Bachmann *et al.* (1993), using an *in situ* hybridisation technique, has localised Epo production to peritubular fibroblasts in the kidneys of anaemic mice. This was recently confirmed by Maxwell *et al.* (1994) who studied the expression of the Epo gene in transgenic mice.

In summary, conflicting opinions still exist with respect to the site of the synthesis of Epo in the kidney. Most authors favour a peritubular origin, which could either be endothelial cells or fibroblasts, but evidence for a tubular origin is also available.

Epo production in the liver has been found to occur in hepatocytes (Koury *et al.*, 1991; Maxwell *et al.*, 1994) as well as in nonparenchymal cells, known as Ito cells (Maxwell *et al.*, 1994). Macrophages, particularly those found in the bone marrow, have also been reported to manufacture Epo (Rich and Kubanek, 1982).

1.5 REGULATION OF ERYTHROPOIETIN SYNTHESIS

It is an established fact that various forms of hypoxia, eg., hypobaric hypoxia, anaemia, or carbon monoxide poisoning, lead to an increase in Epo secretion from the kidney. In addition, it has been shown that certain forms of hypoxia, i.e., anaemia, lead to a significant increase in Epo mRNA (Bondurant and Koury, 1986; Schooley and Mahlman, 1972). This shows that the hypoxia-induced increase in Epo formation is transcriptionally regulated (Schuster *et al.*, 1989; Bauer, 1986). The exact mechanism by which hypoxia triggers the increased production of Epo is, however, still unknown.

The existence of possible "second messengers" in the Epo regulatory pathway remains paradoxical. Administration of

cyclic adenosine monophosphate (cAMP) to mice increased Epo production and RBC mass (Rodgers *et al.*, 1975); prostaglandin E_1 (PGE_1) raised cAMP and Epo concentrations in isolated perfused dog kidneys (Rodgers *et al*, 1975); and PGE_2 increased Epo levels and erythropoiesis in mice (Gross *et al.*, 1976). Adenosine, when perfused through isolated dog kidneys, was also found to increase Epo production (Paul *et al.*, 1988). Renal artery constriction caused increases in PGE and Epo blood levels in dogs; and indomethacin, which inhibits PGE formation, prevented increased Epo production after hypoxia (Mujovic and Fisher, 1975). On the basis of these scattered experiments, many of which were done *in vivo* with multiple variables, it has been proposed that renal hypoxia may result in the release of PGE, which in turn could increase renal cAMP and, thereby, the synthesis of Epo. Cyclic adenosine monophosphate could act through a reduction in the intracellular concentration of Ca^{2+} because cobalt, which stimulates Epo production (Goldwasser *et al.*, 1958), is a general inhibitor of calcium channels. Verapamil and diltiazem, which block calcium entry, have also been shown to enhance Epo production (McGonigle *et al.*, 1987). Recently, Faquin *et al.*(1993) showed that activators of protein kinase C (PKC), such as phorbol myristate acetate (PMA), inhibited Epo production in Hep3B cells and suggested that hypoxia-induced Epo secretion may be negatively regulated by activators of a PKC-mediated pathway.

It is clear that much more work needs to be done before the mechanism that leads to increased renal Epo production is fully understood.

1.5.1 INITIATION OF EPO mRNA TRANSCRIPTION BY HYPOXIA

The chemical transducer for hypoxia is difficult to study in primary renal or hepatic cells because of the small percentage

of Epo-producing cells in these organs and the lack of techniques for purification of these cells. However, cell lines have been screened for Epo production in response to hypoxia and several have been found (Goldberg *et al.*, 1988; Lacombe *et al.*, 1987). The HepG2 and Hep3B cell lines constitutively produce Epo (Goldberg *et al.*, 1988). Hep3B cells, in particular, have one of the largest responses to hypoxia, $CoCl_2$, $NiCl_2$ and $MnCl_2$ (Goldberg *et al.*, 1988, 1989). Barely detectable mRNA for Epo at room air oxygen levels is greatly increased by exposure to 2% oxygen or 50 to 100 µmol/l $CoCl_2$. Because cobalt, nickel, or manganese can be substituted for iron in haem and, in the case of haemoglobin, lock the protein into a deoxy conformation like that of the ferrohaem protein with low oxygen, it has been proposed that a haem protein may regulate Epo production by acting as an oxygen sensor (Goldberg *et al.*, 1988; Porter and Goldberg, 1993). Figure 1.1 depicts the currently proposed model for the oxygen sensor.

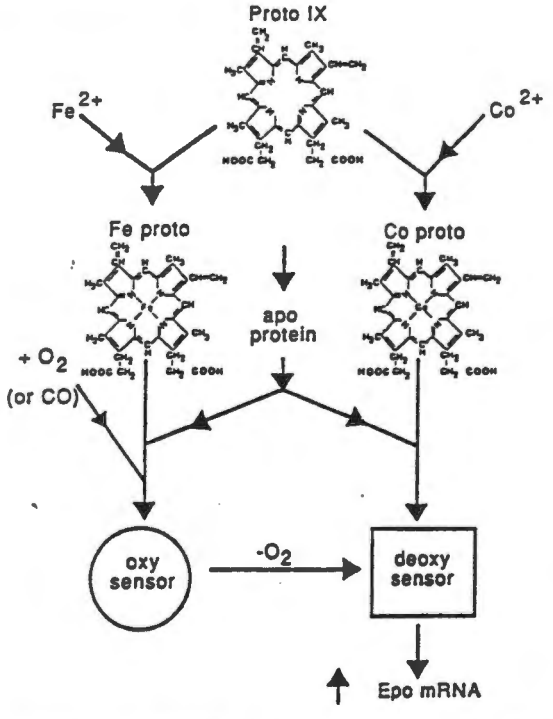


Figure 1.1 Proposed model for the oxygen sensor. This model also suggests a mechanism by which cobalt may mimic the deoxy state and thereby stimulate Epo production. (Adapted from Porter and Goldberg, 1993).

The current conceptual framework of the function of the oxygen sensor that governs Epo formation is characterized by three major features:

1) The oxygen sensor is thought to be mainly located in the kidney itself (Bauer, 1987). However, the extent to which extrarenal humoral or neural factors modulate its function has yet to be quantified.

2) The parameter perceived by the sensor is the venous pO_2, because this variable changes when the oxygen transport capacity of the blood increases or decreases (Kurtz *et al.*,1988).

3) The function of the oxygen sensor is probably related to proximal tubular function, since experimental inhibition of sodium reabsorption at the proximal tubular site results in a decreased sensitivity of the oxygen sensor in terms of Epo production (Eckardt *et al.*, 1989).

It is not known by which mechanism the proximal tubule exerts its influence on the adjacent Epo-producing cells. The main question is whether the Epo-producing cells possess an oxygen sensor of their own or if the adjacent tubuli generate biochemical signals upon hypoxia that are transferred to the Epo-producing cells. The trans- or intracellular biochemical events that lead to an oxygen-dependent production of Epo in the kidney are not fully understood and many questions remain to be resolved (Bauer and Kurtz, 1989; Eckardt and Bauer, 1989). It should be noted,however, that HepG2 cells, which are used in this investigation, are capable of regulated Epo expression (Goldberg *et al.*, 1987) and, thus, must possess an oxygen sensor. Given this fact, it seems likely that the kidney Epo-producing cells may also have their own oxygen sensor.

1.5.2 TRANSCRIPTIONAL REGULATION OF THE ERYTHROPOIETIN GENE

The cloning of the human (Powell *et al.*, 1986) and mouse (McDonald *et al.*, 1986) Epo genes has increased the

understanding of the mechanism that regulates Epo production. One approach to studying Epo transcription has been to put a genomic segment containing the entire human Epo gene with 0.4 or 6 kb of 5'-flanking sequence and 0.7 kb of 3'-flanking sequence into mouse zygotes to generate transgenic mice (Semenza *et al.*, 1989). These mice had regulated hepatic Epo transgene expression with increased hepatic expression after hypoxia or $CoCl_2$, but did not have a similar renal transgene Epo response. Thus, sequences responsible for liver expression map within 0.4 kb of 5'-flanking sequences and 0.7 kb of 3'-flanking sequences, and DNA sequences that control inducible kidney expression have not been found within 6 kb 5' or 0.7 kb 3'. In addition, the smaller transgene had expression in diverse other tissues - something which was not generally seen with the larger gene-suggesting that the Epo gene might contain a negative regulatory element between 0.4 and 6 kb 5' to the coding region that restricted expression to the liver and kidney. In a subsequent study, Semenza *et al.* (1991) identified a 256 bp region of the 3' flanking sequence which could bind four or more different nuclear factors or proteins. At least two of these factors could be induced by anaemia in both liver and kidney. This region was later narrowed down to a 50 nucleotide sequence which functioned as a hypoxia-inducible enhancer and to which only a hypoxia-inducible factor (HIF-1) could bind (Semenza *et al.*, 1992). These studies indicate that control of Epo expression is complex with oxygen-sensitive, tissue-specific, and negative regulatory sequences (Krantz, 1991).

Another approach has been to make plasmid constructs, containing selected potential transcription control sequences 5' and 3' to a reporter gene, and to examine the transcription efficiency of the reporter gene in expression assays after transfection of the plasmid constructs into appropriate mammalian cells. Goldberg *et al.* (1989) have used growth hormone (GH) as the reporter gene. Their construct contained a 1192 bp *Hin*dIII-*Xba*I fragment that extended from 378 bp 5' to the cap site of the Epo gene, through the first intron attached 5' to the GH coding sequence. In addition, a 255 bp *Acc*I-*Bgl*II fragment, that extended 67 bp upstream from the Epo termination codon and covered most of the 3'noncoding region of homology, was added 3'to the GH sequence. The plasmid DNAs were transfected by electroporation into Hep3B cells, which were then exposed to $CoCl_2$ or 1% oxygen.

Measurement of GH in the cell medium indicated stimulated GH expression, which was reduced in the presence of carbon monoxide, as is the case with endogenous Epo synthesis by Hep3B cells. These results show oxygen regulatory sequences within the above regions of the human Epo gene.

Costa-Giomi *et al.* (1990) and Caro *et al.* (1989) have taken yet another approach and prepared nuclear extracts from hypoxic Hep3B cells to determine if they enhance the transcription of the Epo gene. These extracts were added to an *in vitro* transcription system using a DNA template construct consisting of a *Hin*dIII-*Eco*RI fragment, extending from 380 bp 5' to 600 bp 3' of the Epo gene, in a *p*UC8 vector. This fragment was transcribed to a greater extent in the presence of extracts from hypoxic nuclei compared to extracts from normal nuclei. The nuclear extracts from hypoxic Hep3B cells appeared to contain functional transacting factors that specifically stimulated transcription of the Epo gene and these factors appeared to interact with sequences in the above genomic fragment that conferred responsiveness to hypoxia in Hep3B cells.

Beru *et al.* (1990) used a double-stranded deoxyoligonucleotide, corresponding to region -61 to -45 relative to the start site of transcription of the mouse Epo gene, in DNA mobility shift assays. In kidney extracts they found one protein and at least one ribonucleoprotein that bound to this oligonucleotide. Four RNA species also showed specific binding to the oligonucleotide, suggesting that recognition of the oligonucleotide by ribonucleoprotein was mediated by the RNA component. In nuclear extracts of kidneys from stimulated animals, the amount of the two largest RNA species was reduced relative to control. This suggested that the ribonucleoprotein, which contained the down-regulated RNA species, may have been a negative transcriptional factor and that activation of the Epo gene by cobalt salts may involve decreased binding of this factor, thus allowing

transcription to proceed.

Finally, Rondon *et al.* (1991) reported that hypoxia up-regulates the activity of a novel erythropoietin mRNA binding protein, suggesting that mechanisms exist for the post-transcriptional regulation of the Epo gene. In order to determine if Epo mRNA specific binding proteins existed, they probed cytosolic lysates from Hep3B cells and mouse tissues with radiolabelled Epo RNA. A cytosolic protein that binds specifically to Epo RNA was identified in the Epo-producing, hepatoblastoma Hep3B cell line by gel mobility shift assays. This protein was identified in both normoxic and hypoxic cells and found to be bound specifically to a 120 bp fragment of the 3'-untranslated region of Epo mRNA. Ultraviolet light cross-linked Epo RNA-protein complexes, migrated as two bands of 70 and 135-140 kD on sodium dodecyl sulfate-polyacrylamide gels. Binding activity was markedly increased in brain and spleen lysates from mice subjected to 24 h of hypoxia. They concluded that the post-transcriptional regulation of Epo expression in response to hypoxia may, in part, be due to the interaction of Epo RNA with its specific binding protein.

All these methodologies should provide a means for characterization of the controlling sequences, as well as the transacting elements necessary for Epo synthesis, but much more work is still required for precise determination.

1.6 SITE OF ACTION OF ERYTHROPOIETIN

The pluripotential haematopoietic stem cell gives rise to specific erythroid progenitor cells that respond to Epo. These have been termed the colony-forming unit-erythroid (CFU-E) and the burst-forming unit-erythroid (BFU-E) (Stephenson *et al.*, 1971; Tepperman *et al.*, 1974; Gregory *et al.*, 1978; Eaves and

Eaves, 1984). The CFU-E is a rapidly dividing cell which is highly responsive to small concentrations of Epo (Gregory *et al.*, 1978; Ogawa *et al.*, 1977) and gives rise to erythroblast colonies (Stephenson *et al.*, 1971; Tepperman *et al.*, 1974). The BFU-E is a much more immature cell that divides less frequently (Gregory *et al.*, 1978). This cell requires another growth factor such as IL-3 or granulocyte-macrophage colony stimulating factor (GM-CSF) plus Epo (Emerson *et al.*, 1985, 1988). These cells are part of a continuum of development from the earliest BFU-E to the latest CFU-E, with intermediate cells termed mature BFU-E. Figure 1.2 is a schematic outline of erythropoiesis.

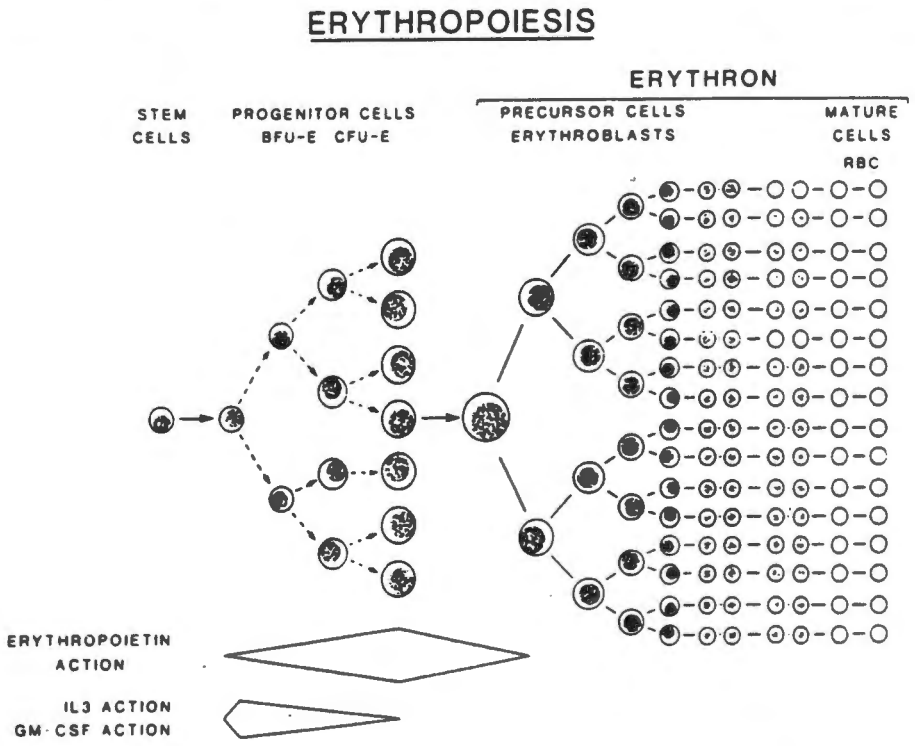


Figure 1.2 Schematic representation of erythropoiesis. (Adapted from Erslev, 1990)

The predominant measurable effects of Epo on early erythroid progenitor cells (BFU-E) are primarily proliferative (Dessypris and Krantz, 1984). As these cells mature and reach the stage of the CFU-E to proerythroblast, differentiation events become much more prominent and, at the orthochromatic erythroblast stage, proliferation ceases. Studies on the effect of Epo on mouse

bone marrow cells infected with the Friend virus that produces anaemia (FVA) (Koury *et al.*, 1982), or thiamphenicol and phenylhyrazine-treated animals (Nijhof *et al.*, 1987), have demonstrated that Epo induces an increase in the synthesis of mRNA and this is closely followed by the induction of alpha- and beta-globin gene transcription. Previous studies have shown that induction of RNA synthesis constitutes one of the earlier biochemical events, following exposure of erythroid cells to Epo, and that stimulation of RNA synthesis is independent of DNA synthesis (Gross and Goldwasser, 1970). Other Epo-induced differentiation events include increased iron uptake, haemoglobin synthesis, and the appearance of erythrocyte membrane proteins (Koury *et al.*, 1982; Gross and Goldwasser, 1970; Hanspal *et al.*, 1991). Thus, Epo exerts profound effects on erythroid cell development and on their terminal differentiation.

The mode of action of the hormone could also be to prevent programmed cell death (apoptosis), since in the absence of Epo, erythroid progenitor cells were found to accumulate DNA cleavage fragments characteristic of those found in apoptosis (Koury and Bondurant, 1990).

Besides the erythroid cells, the only other haematopoietic cells that were found to be stimulated by Epo were the megakaryocytes (MK) and their progenitors (Tsukuda *et al.*, 1992). In murine marrow, megakaryocyte colony formation could be induced by Epo, but required the presence of an unknown serum factor. Neutrophil production in newborn rats was reported to be diminished following the administration of high doses of Epo (Christensen *et al.*, 1991).

1.7 ERYTHROPOIETIN RECEPTOR

The availability of purified recombinant erythropoietin and the development of techniques for obtaining almost pure populations of Epo-responsive cells (Nijhof and Wierenga, 1987) allowed studies on the mechanism of attachment of Epo to specific membrane molecules, the Epo receptors. Epo receptors were initially detected on murine erythroid cells infected by FVA

(Krantz and Goldwasser, 1984). FVA-infected splenic erythroid cells were found to have approximately 800 to 1000 receptors per cell of which 300 had a high affinity for Epo (kd 0.09 nM), while the remainder had a lower affinity (kd 0.57 nM) (Sawyer *et al.*, 1987). Purified human erythroid cells that were generated *in vitro* from circulating immature erythroid progenitors were also found to express two classes of high- and low-affinity Epo receptors on their membranes, with an overall density of 1050 receptors per cell (Fukamachi *et al.*, 1987; Sawada *et al.*, 1989).

Following binding of Epo to its receptor, the hormone is rapidly endocytosed and degraded (Sawyer *et al.*, 1987). The physiological significance of high- versus low-affinity Epo receptors is not fully understood. It is likely that only the high affinity form is responsible for the biologic effect of Epo, as few low-affinity receptors are expected to be occupied at physiological concentrations (Landschulz *et al.*, 1989; Sawyer *et al.*, 1987).

The density of Epo receptors on normal human erythroid progenitor cells appears to correlate with their responsiveness to, and dependence on, this hormone. Erythroid cells at the stage of the CFU-E to the proerythroblast seem to have the highest density of receptors on their membranes. As the cell matures, the number of Epo receptors decreases and eventually they disappear at the stage of the orthochromatic erythroblast (Sawyer and Koury, 1987; Landschulz *et al.*, 1989). In erythroid cells earlier than the CFU-E, such as the erythroid burst forming unit (BFU-E), preliminary studies indicate that the number of Epo receptors is very small and increases to the density found at the CFU-E stage within the first 4 days of culture (Mufson and Gesner, 1987).

Studies on the structure of Epo receptors have demonstrated two bands of 100 and 85 kD by SDS-polyacrylamide gel electrophoresis

(Sawyer *et al.*, 1987). Peptide mapping of proteolytic fragments of these two peptides showed a very similar, if not identical, pattern (Sawyer, 1989). Both human (Jones *et al.*, 1990) and murine (D'Andrea *et al.*, 1989) Epo receptor genes have recently been cloned. The human gene encodes a 508 amino acid molecule, including a 24 residue signal peptide, a 226 amino acid external domain, a transmembrane spanning region of 22 amino acids, and a cytoplasmic domain of 236 amino acids. There is an 80% overall homology between the human and murine erythropoietin receptor sequences (Ehrenman and St John, 1991). This single gene can generate both high- and low-affinity binding sites on transfected cells (D'Andrea *et al.*, 1989).

1.8 SIGNAL TRANSDUCTION

Once the Epo-receptor-complex is endocytosed, signals must be transduced to the nucleus which will initiate proliferation and differentiation of the target cell. Despite the availability of the cloned receptor, the biochemical nature of the growth-regulating signals has remained poorly understood. Before cloning of the receptor cDNA, it was shown that Epo induces a rapid Ca^{2+} influx in erythroid cells infected with the Friend virus (Sawyer and Krantz, 1984) and in immature erythroblasts (Miller *et al.*, 1988). By contrast, mouse CFU-Es did not show a similar increase in intracellular Ca^{2+} (Imagawa *et al.*, 1989). In addition, neither cAMP (Tsuda *et al.*, 1989), nor cyclic guanosine monophosphate (White and George, 1981), seem to act as second messengers. Lipoxygenase products of arachidonic acid metabolism have been considered essential for differentiation of erythrocytic progenitors (Beckman *et al.*, 1987; Mason-Garcia and Beckman, 1991). However, clear proof that eicosanoids are mediators of the effect of Epo is lacking.

The Epo receptor which lacks intrinsic kinase activity, couples ligand binding to phosphorylation by association with the cytoplasmic protein tyrosine kinase Janus kinase 2 (Jak2)

(D'Andrea *et al.*, 1989; Witthuhn *et al.*, 1993). Latent cytoplasmic proteins termed STATs (signal transducers and activators of transcription) also become activated, presumably by the Jaks, through phosphorylation of tyrosine residues. The activated STAT proteins are translocated to the nucleus where they bind to specific sequences (response elements) and stimulate transcription (Darnell *et al.*, 1994). Tyrosine kinase activity of cellular proteins is, thus, involved in a signalling pathway triggered by Epo. Some of the intracellular proteins include an 85 kD regulatory subunit of phosphatidylinositol 3-kinase (Damen *et al.*, 1993), the c-*fps/fes* proto-oncogene product ($p92^{c\text{-}fos}$), nonreceptor tyrosine kinase (Hanazono *et al.*, 1993), and the c-*raf* proto-oncogene product, Raf-1 (Carroll *et al.*, 1991). Exposure of UT-7 Epo responsive megakaryoblastic cells to Epo, also induced tyrosine phosphorylation of several proteins (Komatsu *et al.*, 1992). In fact, the Epo receptor itself becomes phosphorylated upon binding to its ligand (Linnekin *et al.*, 1992).

The cumulative data suggest that the Epo receptor undergoes phosphorylation in response to Epo, which in turn induces phosphorylation of other cytoplasmic or membrane proteins. The precise sequence of events that eventually culminate in a growth signal, as well as the counter-signals that turn the signal off, remain to be elucidated.

1.9 ASSAYS FOR ERYTHROPOIETIN

The concentration of Epo in plasma or serum can be measured by four general techniques: *in vivo* bioassay, *in vitro* bioassay, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). The *in vivo* bioassay measures the biologic activity of Epo in the intact animal and it is considered the reference assay against which the other assays are compared. Epo measurements are usually expressed in units or milliunits of the reference Standard B prepared under the auspices of, and distributed by, the World Health Organisation. The Standard B was prepared from urine of anaemic patients and has a specific activity of about 5 units/mg (Caro and Erslev, 1988). A unit

was originally defined as the erythropoietic activity elicited in rats after the injection of 5 μM of $CoCl_2.6H_2O$.

The *in vivo* bioassay utilizes polycythemic mice by either hypertransfusion or pre-exposure to hypoxia for a period of two weeks (Alippi *et al.*, 1983a,b; Dunn and Lange, 1980; Caro and Erslev, 1988). These polycythemic animals have a very low endogenous level of Epo and can be utilized for the measurement of exogenously injected Epo. The effect of Epo on red cell production is measured by the incorporation of ^{59}Fe into circulating erythrocytes. The lowest limit of sensitivity with this assay is about 50 mU and it will not detect the erythropoietic activity found in normal plasma.

Numerous *in vitro* bioassays have been designed and shown to be of use in measuring Epo titres. These assays are based on short-term cultures of erythroid tissue derived from bone-marrow, spleen or foetal livers (Sakat *et al.*, 1987; Caro and Erslev, 1988). The erythropoietic effect is measured by the incorporation of ^{59}Fe into newly formed haem, the uptake of ^{3}H-thymidine as a measure of cellular proliferation, or the stimulation of CFU-E formation in semisolid media. The main problem with *in vitro* techniques is the presence of some poorly defined inhibitors in human sera and the nonspecific stimulatory effect of other growth factors in these *in vitro* systems.

The successful purification of human Epo has allowed the development of sensitive and specific radioimmunoassays. Recombinant human Epo (rhu-Epo) eventually replaced pure native Epo in the RIA (Lertora *et al.*, 1975; Nielsen, 1988). These assays utilize radiolabelled pure Epo and antibodies developed against either pure or impure Epo, Epo oligopeptides, or fusion proteins. Radioimmunoassays have the potential of measuring nonbiologically active asialo-Epo. However, there have been no confirmed reports on significant discordance between bioassays

and radioimmunoassays. The lower limit of sensitivity of the RIA is about 2 to 4 mU/ml and it is quantitative. The primary disadvantage of the RIA is the use of radioactively labelled Epo (Caro and Erslev, 1988).

The combined availability of recombinant human Epo and monoclonal antibodies has recently led to the development of enzyme-linked immunosorbent assays. One assay, described by Noe *et al.* (1992), utilizes a monospecific polyclonal antibody raised in rabbits against rhu-Epo. The procedure involves coating the wells of a microtitre plate with anti-Epo, adding the Epo standards and samples and then leaving them to bind to the catching antibody. This is followed by the addition of the same antibody that has been biotinylated. Finally, anti-biotin conjugated to alkaline phosphatase is added and the enzyme reaction developed and read spectrophotometrically at 405 nm. The assay is rapid, reproducible and, above all, quantitative, specific, and sensitive with a minimum detectable concentration of 1.2 to 2 mU/ml of serum.

The clinical utility of Epo assays has not yet been proved. Measurement of Epo levels may be helpful in distinguishing primary from secondary polycythemia and may be able to identify cases of anaemia with a poor Epo response, as in patients with chronic renal disease.

1.10 PATHOPHYSIOLOGY OF ERYTHROPOIETIN

Excessive production of Epo leads to secondary erythrocytosis. It may be induced by hypoxia or arise autonomously. In contrast, polycythemia vera is caused by erythropoietin-independent growth of erythrocytic progenitors from abnormal stem cell clones (Cashman *et al.*, 1983; Dai *et al.*, 1991)

With peripheral oxygen delivery being the main regulatory parameter, the production of erythropoietin increases at lowered arterial oxygen tension. Both natives and visitors at high altitude, exhibit erythrocytic hyperplasia of their bone marrow, erythrocytosis, and increased blood haemoglobin concentration (Lenfant and Sullivan, 1971). Even though the action of Epo is protective against anaemia, its benefits in terms of improved oxygen transport are outweighed by rheological and haemodynamic disadvantages in hypoxaemia (Editorial, 1989). Peripheral resistance and work load of the heart are increased. In particular, blood flow is obstructed in the lungs where hypoxaemia causes vasoconstriction. Erythrocytosis, congestive heart failure, and dyspnoea are the most serious manifestations of chronic mountain sickness.

In contrast with altitude hypoxaemia, chronic respiratory failure at sea level seldom leads to severe erythrocytosis (Fried *et al.*, 1970). The percentage of patients reported with elevated plasma Epo, has varied between 21 and 85% (Guidet *et al.*, 1987; Miller *et al.*, 1981). Intermittent nocturnal hypoxia in patients with obstructive sleep apnoea is not accompanied by elevated serum erythropoietin or erythrocytosis (McKeon *et al.*, 1990).

Hypoxia-induced erythrocytosis occurs as an inherited disorder, in association with mutant haemoglobins or red blood cell 2,3-bisphosphoglycerate deficiency, causing high blood oxygen affinity (Adamson and Finch, 1975). Inherited erythrocytosis was also recently documented in a family with a truncated Epo receptor due to a single mutation in their receptor gene sequence (De La Chapelle *et al.*, 1993). Elevated plasma Epo without manifest erythrocytosis was observed in subjects with congestive heart failure, and this elevation was abolished by treatment with the angiotensin-converting enzyme-inhibiting drug, enalapril (Fyhrquist *et al.*, 1989).

Certain tumours cause erythrocytosis secondary to the production of Epo by the neoplastic cells. Thorling (1972) measured increased plasma Epo bioactivity in 19 of 25 patients with renal carcinoma-associated erythrocytosis. Da Silva *et al.* (1990) measured high serum levels of Epo by RIA in three patients who also presented with renal adenocarcinoma and polycythemia. Patients with liver carcinoma (Kew and Fisher, 1986), haemangioblastoma (Trimble *et al.*, 1991) and meningioma (Bruneval *et al.*, 1993) associated with erythrocytosis, also had elevated serum levels of Epo. In some cases, plasma Epo, haematocrit, and blood haemoglobin concentration normalised after the tumour-bearing kidney was removed and later increased again, heralding tumour recurrence (Murphy *et al.*, 1976, Bruneval *et al.*, 1993).

Erythropoietin deficiency is the primary cause of the anaemia in chronic renal failure (Eschbach, 1984). The hypoproliferative anaemia associated with uraemia is usually normochromic and normocytic (Eschbach *et al.*, 1980, 1984). Its severity increases with the impairment of renal function regardless of the underlying disease (Zappacosta *et al.*, 1982). Switching from haemodialysis to continuous ambulatory peritoneal dialysis occasionally improves the anaemia (Zappacosta *et al.*, 1982). Still, almost all of the patients in the later stages of renal failure were anaemic until recombinant human Epo became available for therapy (Winearls *et al.*, 1986; Casati *et al.*, 1987; Adamson and Eschbach 1989).

Acute (Chandra *et al.*, 1988) or chronic (Muto *et al.*, 1987) hypoxic stress may result in greatly increased plasma Epo activity in patients with chronic renal failure. This finding indicates that the capacity to produce Epo is not necessarily abolished in uraemia. Instead the mechanisms that control the synthesis of Epo may operate at a lower set point in some of the patients with chronic renal failure (Chandra *et al.*, 1988).

Mild anaemia frequently develops in patients with chronic infections, autoimmune diseases, or malignancies (Hansen, 1983; Cartwright and Lee, 1971). The factors which characterized this so-called anaemia of chronic disorders include insufficient iron

availability, decreased total iron-binding capacity of the plasma, reduced saturation of transferrin with iron, decreased bone marrow sideroblasts, normal or increased reticuloendothelial iron, inhibition of the proliferation of erythrocytic progenitors by inflammatory cytokines, increased haemolysis, and bleeding (Cartwright and Lee, 1971; Cash and Sears, 1989; Birgegard, 1989). In addition, plasma Epo levels may also be inappropriately low for the degree of anaemia (Baer *et al.*, 1987; Spivak *et al.*, 1989; Miller *et al.*, 1990).

The immune system may have a modulating role on the production of Epo. Infectious and inflammatory processes induce the production of immunomodulatory peptides by leucocytes, tissue macrophages, fibroblasts, and endothelial cells. In turn, these peptides or cytokines are involved in the control of the immune reactions and of haemopoiesis (Hausen *et al.*, 1991; Fribbe and Wllemze, 1991; Arai *et al.*, 1990). Regarded as important mediators of the anaemia of chronic disorders, interleukin-1 (Johnson *et al.*, 1989) and tumour necrosis factor-*alpha* (Johnson *et al.*, 1990; Broxmeyer *et al.*, 1986), have been shown to lower red blood cell production *in vivo*. The influence of immunomodulatory peptides on the production of Epo remains to be clarified and is the primary aim of this investigation.

1.11 CLINICAL APPLICATIONS

The successful cloning and expression of the human Epo gene has made recombinant human Epo (rhu-Epo) available, not only for biological investigation, but also for therapeutic application. The therapeutic efficacy of rhu-Epo was first evaluated in patients with end-stage renal disease with severe anaemia. The results of the initial phase I and II clinical trials with rhu-Epo were reported by Eschbach and co-workers (1987). A dose- and time-related increase of reticulocytes and haematocrit was

recorded, and correction of anaemia and disappearance of blood transfusion requirements was achieved in the majority of patients receiving effective dosages. Elimination of transfusion requirements represents a major improvement for such patients, who thus avoid the risk of iron overload, exposure to infectious agents, and alloimmunization (Foa, 1991). These encouraging results have been confirmed by other studies which all showed the efficacy of the recombinant hormone in this type of anaemia (Casati *et al.*, 1987; Winearls *et al.*, 1986).

Recombinant human Epo is today approved in more than fifty countries, including South Africa, for the treatment of the anaemia associated with chronic renal failure. In addition, it has also been evaluated in other types of anaemia. Patients with the anaemia of rheumatoid arthritis showed excellent haematologic responses to recombinant Epo (Pincus *et al.*, 1990). So did patients with the anaemia of cancer (Abels, 1992) and infants with the anaemia of prematurity (Halperin *et al.*, 1990; Phibbs *et al.*, 1992). AIDS patients, with anaemia associated with HIV infection or zidovudine therapy, have also benefitted from treatment with rhu-Epo (Fischl, 1990).

Development or worsening of hypertension is generally considered the most serious adverse effect of the treatment with rhu-Epo (Raine, 1988). Its frequency appears to be higher in patients with a previous history of hypertension (Casati *et al.*, 1987).

In elective surgical procedures, the transfusion of preoperatively collected autologous blood eliminates the infectious and immunological hazards of donor blood (Foa, 1991). Despite the development of anaemia, the level of endogenous Epo increases only slightly in response to repeated phlebotomy in autologous blood donors (Kickler and Spivak, 1988). A multicentre study in humans confirmed the ability of Epo treatment to facilitate autologous blood donation (Goodnough *et*

al., 1989). Aside from the possibility of improving autologous blood donation, the severity and the duration of postoperative anaemia might be lessened by the application of recombinant human Epo some days before surgery (Abels and Rudnick, 1991).

1.12 HYPOTHESIS AND OBJECTIVES

Chronic inflammatory diseases are frequently accompanied by a persistent anaemia, which is now widely believed to be due to the suppression of bone marrow erythropoiesis by inflammatory cytokines (Means and Krantz, 1992). Several investigators have also postulated that a compromised erythropoietin response may contribute to the protracted anaemia (Miller *et al.*, 1990; Spivak *et al.*, 1989; Baer *et al.*, 1987). This inappropriate or blunted Epo response is purportedly also mediated by cytokines. The cytokines, interleukin-1 (IL-1) and tumour necrosis factor-*alpha* (TNFα), were found to inhibit Epo secretion by Epo-producing hepatoma cell lines in culture (Jelkmann *et al.*, 1990; Faquin *et al.*, 1992). No *in vivo* studies have yet been carried out to confirm these findings.

Both IL-1 and TNFα, together with interleukin-6, are mediators of the hepatic protein response during acute inflammation in mammals (Andus *et al.*, 1988; Mortensin *et al.*, 1988). Since acute inflammation is often accompanied by a mild anaemia (Schreiber *et al.*, 1989), the accompanying Epo response may also be compromised.

It is hypothesized, therefore, that Epo synthesis is antagonised by cytokines elaborated during acute and chronic inflammation. This results in a persistence of the cytokine-induced anaemia, which will only resolve once the underlying inflammatory reaction has subsided.

Verification of this hypothesis would require:

1. Determining whether inflammatory cytokines do indeed inhibit Epo synthesis *in vitro* and, if so, elucidating the mechanism of such inhibition.

2. Studying the synthesis of Epo in animal models of acute and chronic inflammation and comparing it to that in normal controls and controls with a comparable degree of experimental anaemia.

CHAPTER TWO

CYTOKINE-MEDIATED INHIBITION OF *IN VITRO* ERYTHROPOIETIN SYNTHESIS

2.1 INTRODUCTION

The pathogenesis of the anaemia of chronic disorders (ACD) is now widely accepted to be due to cytokines secreted by activated monocytes and macrophages during inflammation (Means and Krantz, 1992; Hausen *et al.*, 1991). Interleukin-1 and tumour necrosis factor-alpha are the two main cytokines implicated in a reduced erythropoiesis. The growth in culture of human erythroid precursor cells, known as burst forming units-erythroid (BFU-E) and colony forming units-erythroid (CFU-E), was significantly inhibited by low doses of TNFα (Broxmeyer *et al.*, 1986; Roodman *et al.*, 1987). Chronic administration of TNFα also suppressed erythropoiesis in mice (Johnson *et al.*, 1989, 1990) and rats (Moldawer *et al.*, 1989). Interleukin-1 was found to antagonize the proliferative effect of Epo on mouse erythroid precursor cells in culture (Clibon *et al.*, 1990).

Recently it was proposed that cytokines may also reduce the increased Epo biosynthesis that occurs in response to anaemia. A decreased Epo response was reported in patients with the anaemia of cancer (Miller *et al.*, 1990) and in anaemic patients infected with the human immunodeficiency virus (Spivak *et al.*, 1989). These findings appear to support the contention that Epo synthesis may be compromised during chronic disease. Cytokines usually contribute positively to the host's defenses against pathogenic agents that precipitate inflammatory responses (Arai

et al., 1990; Offner *et al.*, 1990). However, a sustained and prolonged stimulation of inflammatory cells (eg. macrophages), as would be the case during chronic inflammation, frequently leads to overproduction of these substances which can result in tissue and/or systemic pathology (Dinarello and Wolff, 1993; de Benedetti *et al.*, 1991; Vassalli, 1992). Failure of the anaemia to become resolved in patients with chronic disease may, therefore, be due in part to a cytokine-mediated inhibition of Epo biosynthesis. This hypothesis was tested by studying the effects of macrophage-conditioned medium (MCM), as well as selected purified products of macrophages, on the *in vitro* production of Epo by a hepatoma cell line that constitutively expresses the hormone. Before reporting on the findings, a summary of the characteristics of each macrophage factor investigated for suppression of Epo synthesis, is given.

2.1.1 INTERLEUKIN-1

Interleukin-1 (IL-1) is a cytokine that is responsible for mediating a variety of processes in host defence, inflammation, and response to injury (Arai *et al.*, 1990; Dinarello *et al.*, 1991). Although initially described as a product of activated macrophages and monocytes, IL-1 is now known to be produced by a variety of cells in response to infection and injury. These include fibroblasts, keratinocytes of the skin, T and B lymphocytes, as well as brain astrocytes and microglial cells (Dinarello *et al.*, 1991). IL-1 consists of two distinct but related molecules: IL-1α and IL-1ß. Both have a similar molecular size, 17 kD; however, IL-1α is acidic with an isoelectric point (pI) of 5.3, while IL-1ß has a pI of 7.2 (Dinarello *et al.*, 1974). Although there is only a 26% amino acid sequence homology and a 45% nucleotide homology between the two forms of IL-1, they have essentially identical biological properties and share the same receptor (Dinarello, 1991).

IL-1 appears to be a primary molecule in inflammatory reactions through its induction of other inflammatory metabolites. It induces production of prostaglandin E_2, collagenase, and phospholipase A_2 (Dejana *et al.*, 1987). In addition to attracting leucocytes into inflamed tissues, IL-1 also causes degranulation of basophils and eosinophils, stimulates thromboxane synthesis in macrophages and neutrophils, and potentiates the activation of neutrophils by chemoattractant peptides (Dinarello, 1988). In contrast to its catabolic activities, IL-1 participates in the reparative process by increasing fibroblast proliferation as well as the synthesis of collagens and glucosaminoglycans (Dinarello, 1988). IL-1 also has the properties of an hepatocyte-activating factor and mediates the induction of acute-phase proteins by the liver (Mortensin *et al.*, 1988; Andus *et al.*, 1988).

IL-1 has also been implicated as a mediator of disease. Fibrosis and deposition of abnormal proteins in tissues appear to be, in part, mediated by IL-1: In rheumatoid joint disease, this contributes to thickening of scar tissue which results in the restriction of joint movement (Dinarello, 1988). The mechanism of IL-1-induced shock appears to be the ability of IL-1 to increase the plasma concentrations of platelet-activating factor, prostaglandin, and nitric oxide. These substances are potent vasodilators and induce shock (Dinarello and Wolff, 1993; Okusawa *et al.*, 1988). Other diseases in which a role for IL-1 has been identified include inflammatory bowel disease (Dinarello and Wolff, 1993), insulin-dependent diabetes mellitus (Dinarello and Wolff, 1993; Corbett *et al.*, 1993), acute and chronic myelogenous leukaemia (Peled *et al.*, 1992), and atherosclerosis (Ross, 1993). IL-1 may also induce anaemia by antagonizing the action of Epo on erythroid precursors (Schooley *et al.*, 1987).

2.1.2 INTERLEUKIN-6

Interleukin-6 (IL-6), along with IL-1, is perhaps the best example of a cytokine that mediates different host responses. It is not only produced by a number of cells including T cells, macrophages, and fibroblasts, but is also involved in the regulation of a number of cell types, including nonhaematopoietic cells (Arai *et al.*, 1990).

On haematopoietic cells and stem cells, IL-6 can stimulate the growth of haematopoietic colonies composed of granulocytes and macrophages (Wong *et al.*, 1988). In addition, IL-6 appears to stimulate the ability of stem cells to respond to IL-3. When IL-6 is combined with other recombinant haematopoietic factors, it can augment responses to these factors or even modify them (Rennick *et al.*, 1989). IL-6 is also a potent inducer of differentiation when added to certain myeloid leukaemia cell lines (Chiu and Lee, 1989). IL-6 also appears to function as a growth factor for certain transformed B cells (Tosato *et al.*, 1988), and as a stimulatory factor for T cells at different stages of differentiation (Hodgkin *et al.*, 1988). IL-6 also mediates the acute phase response by liver cells (Andus *et al.*, 1988; Castell *et al.*, 1989).

Elevated serum levels of IL-6 have been detected in patients with rheumatoid arthritis (Wood *et al.*, 1992; de Benedetti *et al.*, 1991) and in animals with experimental chronic inflammation (Shacter *et al.*, 1992; Utsunomiya *et al.*, 1991).

2.1.3 TUMOUR NECROSIS FACTOR-*alpha*

Tumour necrosis factor-*alpha* (TNFα) caused the necrosis of certain tumours when injected into tumour-bearing animals and

was found to be cytotoxic to a number of transformed cell lines *in vitro* (Carswell *et al.*, 1975; Helson *et al.*, 1975; Philip and Epstein, 1986). The exaggerated production of TNFα *in vivo* can cause profound systemic alterations with potentially life-threatening consequences. Its involvement in septic shock is supported by several lines of evidence. For example, the infusion of TNFα into experimental animals resulted in a syndrome similar to the effects of lipopolysaccharide (LPS) infusion and septic shock (Tracey *et al.*, 1986). In addition, in patients with meningococcal septicaemia, plasma levels of TNFα were predictive of the outcome of the illness; TNFα was detectable in the plasma of 10 of 11 patients who died (Waage *et al.*, 1987), but in only 6 of 68 survivors. Perhaps the most convincing evidence for the role of TNFα in septicaemia is derived from studies in which anti-TNFα antibodies were administered to mice before a lethal dose of LPS (Beutler *et al.*, 1985). The group receiving the TNFα antibodies showed a significantly reduced mortality rate compared with the control group. TNFα, as discussed earlier, has also been shown to induce anaemia *in vivo* (Johnson *et al.*, 1989; Moldawer *et al.*, 1989) probably by inhibiting the proliferation of erythroid precursor cells (Broxmeyer *et al.*, 1986; Roodman *et al.*, 1987).

2.1.4 NEOPTERIN

Neopterin is a pyrazino-pyrimidine compound derived from guanosine triphosphate. It represents an intermediate in the synthetic pathway of biopterin (Smith and Nichol, 1986). Biopterin, itself, is known to serve as an essential cofactor in neurotransmitter synthesis (Kaufmann *et al.*, 1978). Immune responses *in vitro* and *in vivo* are accompanied by increased neopterin levels. *In vitro* release of neopterin from human peripheral blood mononuclear cells was induced by stimulation with alloantigens, virally or chemically modified autologous

cells, or with mitogenic lectins (Huber *et al.*, 1983). *In vivo*, increased neopterin excretion was demonstrated in patients suffering from infections with viruses and intracellular bacterial or protozoal pathogens (Huber *et al.*, 1984), from rheumatoid arthritis (Reibnegger *et al.*, 1986), and from acute cellular graft rejection or graft-vs-host disease (Huber *et al.*, 1983). Elevated neopterin levels were also seen in patients with acquired immunodeficiency syndrome (Fuchs *et al.*, 1984) and in those with certain tumour states (Reibnegger *et al.*, 1986). In these diseases, an increase of neopterin excretion usually precedes the clinical manifestation, and is correlated with the activity of the underlying disease.

It is clear from the discussion above, that high concentrations of IL-1, IL-6, TNFα, and neopterin predominate in the serum of patients with chronic disease. At such high concentrations, agents like IL-1 and TNFα have been shown to cause pathological effects on normal metabolism. Down-regulation of Epo expression during chronic inflammation may, thus, also be a consequence of their action, thereby contributing to the development of ACD. This hypothesis was tested by investigating the effects of these agents, together with MCM, on the *in vitro* biosynthesis of Epo by HepG2 cells, a cell line capable of regulated production of the hormone.

2.2 MATERIALS AND METHODS

2.2.1 REAGENTS

Human recombinant IL-1ß was provided by the Biological Resources Branch of the Biological Response Modifiers Program, Division of Cancer Treatment/NCI, Frederick, Maryland, USA. It had a specific activity of 1.81 units/mg protein with an endotoxin

content of 2.5 Eu/mg protein. Its purity was greater than 99% as determined by SDS-PAGE, isoelectric focusing, and reverse-phase HPLC analysis. Recombinant human IL-6 (specific activity of approximately $2x10^8$ U/mg protein) and TNFα (specific activity greater than $1x10^8$ U/mg protein), were purchased from Boehringer Mannheim (S.A.). Foetal calf serum was obtained from Highveld Biological, and trypsin, thioglycollate broth, phosphate buffered saline (PBS), Hepes buffer, and *alpha*-minimal essential medium (α-MEM) from Gibco. Dexamethasone, neopterin, lipopolysaccharide (LPS), 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma. Sac-cel (donkey anti-rabbit antibody coated cellulose suspension) was obtained from IDS (England). Iodinated ^{125}I-erythropoietin (370 kBq, 10 µg/0.5 ml) was purchased from Amersham (England).

2.2.2 CELLS AND CELL CULTURE

HepG2 cells, a human hepatoma cell line capable of both constitutive and regulated Epo expression (Goldberg *et al.*, 1987; Ueno *et al.*, 1989), were a gift from Dr B B Knowles (Wistar Institute, Pennsylvania, 19104). The cells were cultured in α-MEM, supplemented with 10% FCS (not heat-inactivated), in a humidified atmosphere containing 5% CO_2 at 37°C. Near confluent cultures were passaged once per week by treatment with 0.25% trypsin and 0.02% EDTA, in Ca^{2+}- and Mg^{2+}-free PBS.

2.2.3 PREPARATION OF CONDITIONED MEDIUM

Inflammatory macrophages were induced in five adult male Balb/c mice (±20 g) by an intraperitoneal injection of 2 ml of 3% (w/v) thioglycollate broth. Three days later the macrophages were

collected by peritoneal lavage using PBS containing 2% heat-inactivated FCS. The cells were pooled and centrifuged at 200*g* for 10 min at 4°C. The cells were washed once with α-MEM containing 5% FCS and gentamicin (20 µg/ml). Adherent macrophages were obtained by plating the cells in Falcon plastic 75 cm^2 flasks (Becton Dickinson) for 2 h at 37°C in 5% CO_2/95% air, followed by removal of nonadherent cells by suction. Half of the cultures were incubated with α-MEM, supplemented with 10% FCS and 10 µg/ml LPS, while the other half were incubated with the same medium containing, in addition, 1 µM dexamethasone. The cells were cultured for 18 h under standard conditions at a density of 10^6 cells/ml.

2.2.4 CELL VIABILITY ASSAYS

Since TNFα can lyse sensitive tumour cells (Philip and Epstein, 1986; Helson *et al.*, 1975), cytotoxicity assays were carried out to define a dose range for each compound in which it would be nontoxic to HepG2 cells.

A colorimetric assay, based on the selective ability of living cells to reduce the yellow salt, MTT, to formazan (dark blue product), was used to determine cell viability (van de Loosdrecht *et al.*, 1993). Briefly, HepG2 cells were seeded at $1x10^4$ cells/well in 96-well plates and incubated overnight under standard conditions to permit adherence. The next morning the culture medium was replaced with 200 µl of fresh medium per well, with or without macrophage-conditioned medium (dose range: 0 to 50% of culture volume), recombinant cytokines (0 to 1000 U/ml), or neopterin (0 to 1000 nM). Plates were returned to the incubator for 44 h, whereafter 50 µl MTT (2 mg/ml) was added to each well, followed by a final incubation at 37°C for 4 h. The culture medium was aspirated from the wells, after which the formazan crystals were dissolved in 200 µl DMSO. The absorbance

was recorded directly on a microplate spectrophotometer (Cambridge Technology Inc) at a wavelength of 540 nm. The spectrophotometer was calibrated using wells that contained only DMSO. Doses for each test agent were assayed in replicates of eight and each experiment was repeated thrice. The results of one experiment are presented which were representative of all three. Cell viability was assessed by comparing the absorbance of the formazan product obtained with controls with that of treated cells.

2.2.5 GROWTH CURVES

Growth curves for HepG2 cells in the presence and absence of a fixed concentration of each test agent were carried out. For neopterin the concentration was 1000 nM, and for IL-1ß, IL-6, and TNFα the dose was 1000 U/ml. (These were the maximum doses used for determining the dose-response effect of each agent on HepG2 Epo synthesis). Cells were plated in 60 mm petri dishes at a density of $2x10^5$ cells/dish in 4 ml of standard medium containing the test substance. Viable cells in three cultures were counted with a haemocytometer every alternate day after trypsinization.

2.2.6 DOSE-RESPONSE CURVES

HepG2 cells were plated at $5x10^5$ cells/well in 24-well Falcon culture plates. Following adherence, after overnight incubation, spent medium was replaced by 1 ml of standard culture medium containing a specific dose of test substance. Cells were cultured in this medium for 24 h, after which the medium was transferred to Eppendorf tubes (Lasec), and stored at -40°C for Epo analysis. Cell monolayers were washed with PBS and the cells lysed with 0.5 ml of NP-40 lysis buffer (Appendix). Protein analysis was carried out on a 1 in 10 dilution (with PBS) of the cell lysates using the Bio-Rad

micro-assay kit (Bio-Rad Laboratories, UK). This was done in order to express the concentration of Epo in terms of mass of cellular protein, thereby, compensating for any effects of the test substances on cell growth. Each agent was assayed over a range of concentrations (0 to 1000 U/ml for cytokines, 0 to 40% for MCM, and 0 to 1000 nM for neopterin) in quadruplicate, and each experiment was repeated three times.

2.2.7 CYTOKINE COMBINATION ASSAYS

Synergism amongst macrophage-derived cytokines, especially in the induction of acute phase protein synthesis, has been reported (Andus *et al.*, 1988; Helle *et al.*, 1988). To determine whether synergism also occurs amongst the test agents on Epo synthesis, HepG2 cells ($5x10^5$ cells/well) were incubated for 24 h with single doses (100 U/ml) of each cytokine in the following combinations: IL-1ß and IL-6, IL-1ß and TNFα, IL-6 and TNFα, and finally, IL-1ß, IL-6, and TNFα. Combinations of each cytokine with neopterin (100 nM) and MCM (10%) were also investigated for synergistic effects on HepG2 Epo production. Each combination was assayed in quadruplicate and each experiment was repeated thrice.

2.2.8 ERYTHROPOIETIN ASSAY

An in-house RIA method for Epo, developed in the Haematology Department at UCT, was used. The method is similar to that of Garcia *et al.* (1982) with minor modifications. To 100 µl of sample or standard in 5 ml plastic tubes was added 200 µl of rabbit anti-human Epo antiserum. This was allowed to react for 72 h at 4°C. To this was added 200 µl of radioactively labelled ^{125}I-Epo equivalent to 10 000 cpm. This was incubated for a further 24 h at 4°C. The next step was to add 100 µl of Sac-cel

(donkey anti-rabbit antibody coated cellulose suspension). The mixture was allowed to stand at room temperature for 30 min after which 1 ml of distilled water was added. The tubes were centrifuged for 10 min at 1500*g* and 4°C. Supernatants were decanted and the radioactivity in the pellets was counted with a Crystal II multidetector RIA system (Packard Instruments, USA). Using the logistic fit of the instrument, a standard curve was drawn from which the results were automatically calculated. The dose range of the standard curve was from 0 to 500 mU/ml. The intra-assay coefficient of variation (% CV) was found to be 10.4% and the interassay CV, 9.8%.

2.2.9 STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the means (SEM). One-way analysis of variance was used to analyse the dose-response curves. Differences between doses were compared using multiple range analysis. Comparisons of the effects of single doses of test substances alone and in combination were carried out using Dunnet's *t*-test. A probability (*p*) value less than, or equal to, 0.05 was considered significant.

2.3 RESULTS

2.3.1 CELL VIABILITY

Figure 2.1 represents the effects of the macrophage-derived factors on the viability of HepG2 cells in culture. None of the factors, including macrophage-conditioned medium, had any deleterious effects on HepG2 cell viability over their tested concentration range. Tumour necrosis factor-*alpha*, a known cytotoxin for certain tumour cell lines (Cairns *et al.*, 1991;

Helson *et al.*, 1975), did not induce necrosis in cells over the 48 h time course of the experiment (Figure 2.1c). Neither did MCM (Figure 2.1a), IL-1ß (Figure 2.1b), or neopterin (Figure 2.1d). Interleukin-6 was not tested for cytotoxicity. Studies on IL-6 as a modulator of acute phase protein synthesis by HepG2 cells and in which the concentration range used was the same as in this investigation have been reported (Castell *et al.*, 1989). Viability of the cells in these studies was not affected.

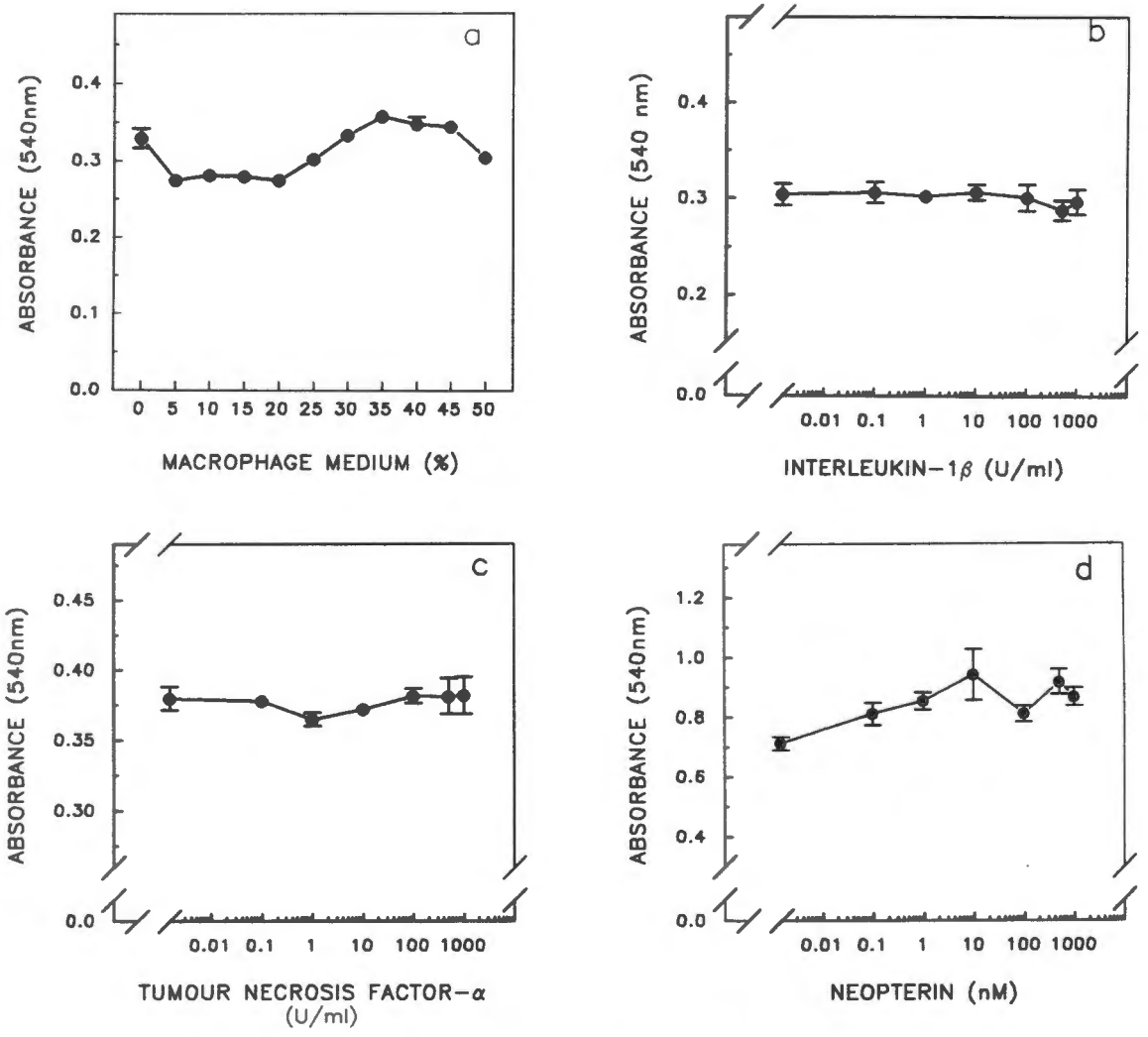


Figure 2.1. The effect of a) MCM, b) IL-1β, c) TNFα, and d) neopterin on the viability of HepG2 cells in culture. Viability of the cultures was determined by assessing their ability to reduce MTT to its formazan product after a 44 h incubation with different doses of the test agents. Data are representative of three independent experiments.

2.3.2 CELL GROWTH

Proliferation of HepG2 cells was generally not affected when cultured with the four macrophage-derived factors. Although the average growth rates of the cells exposed to IL-1ß (Figure 2.2a), IL-6 (Figure 2.2b), TNFα (Figure 2.2c), and neopterin (Figure 2.2d) were lower than for the controls, the differences, especially over the first five days, were not statistically significant.

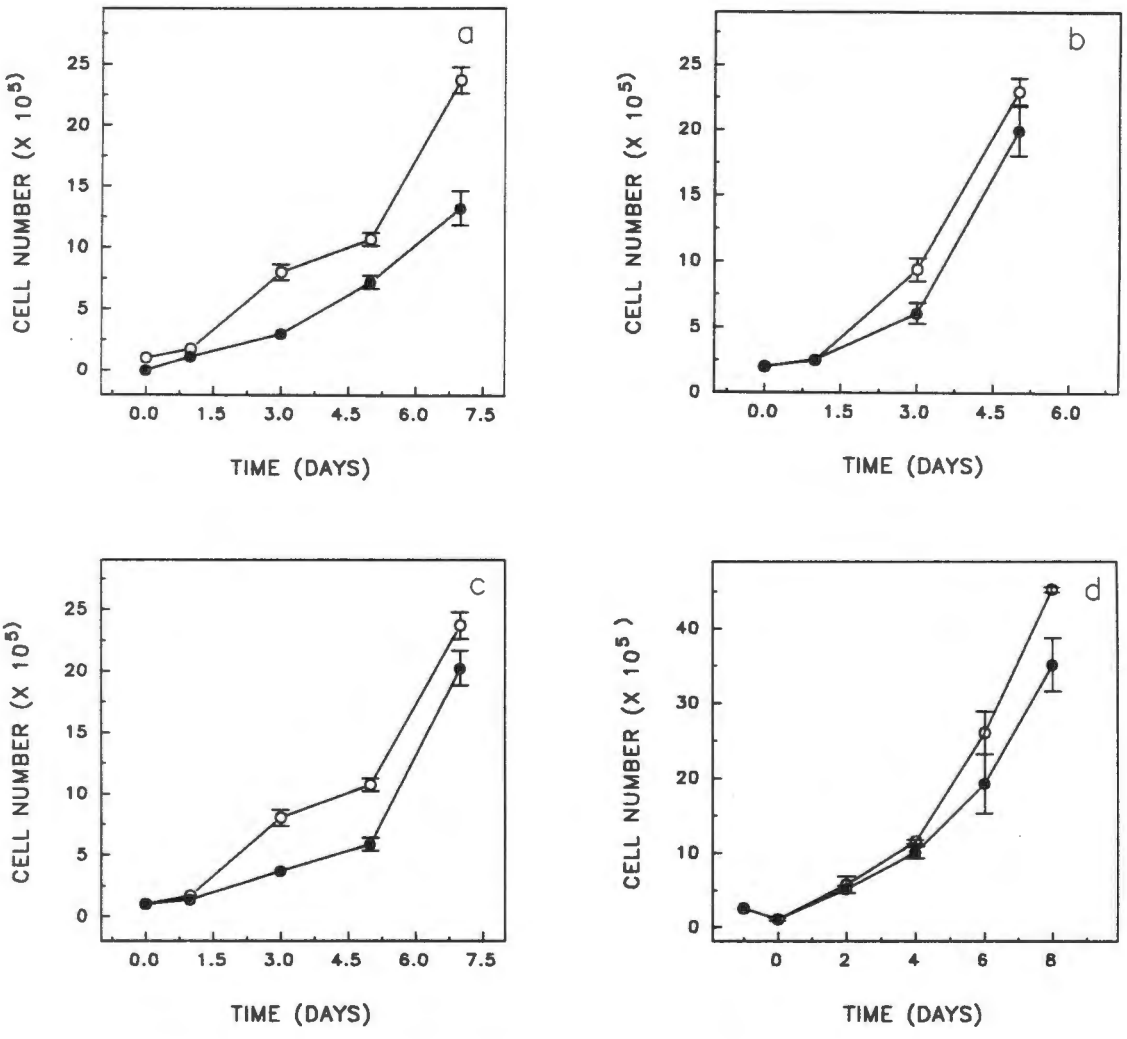
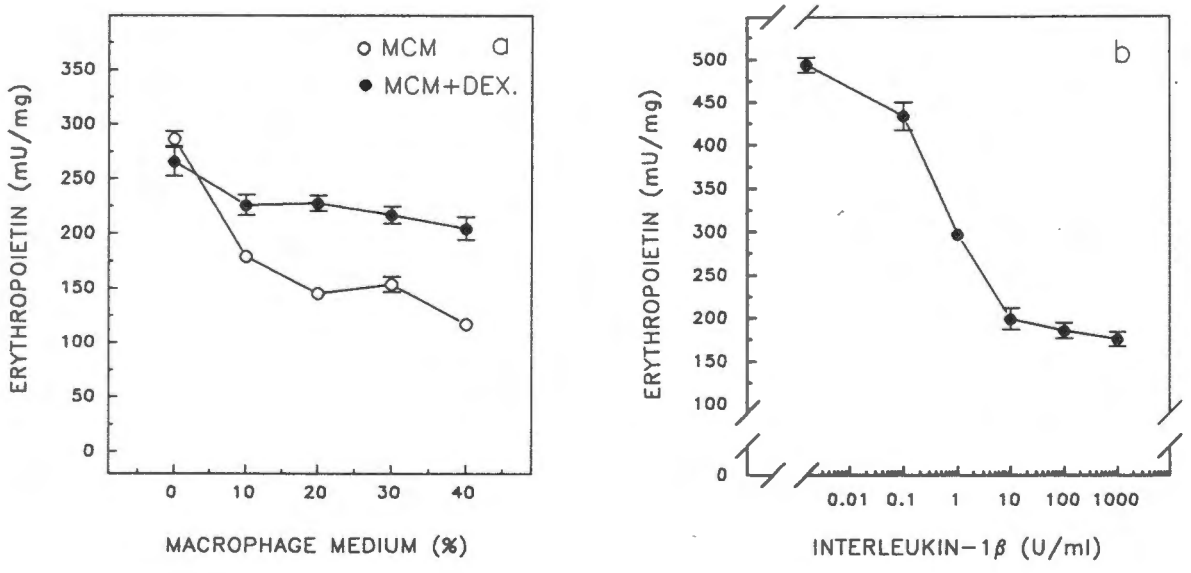


Figure 2.2. The effect of a) IL-1β, b) IL-6, c) TNFα, and d) neopterin on the growth of HepG2 cells in culture. Growth rates were determined by incubating HepG2 cultures with (●) or without (○) a fixed dose of each test agent and counting the viable cells every alternate day.

2.3.3 DOSE-RESPONSES

Conditioned medium obtained from LPS-stimulated macrophages caused a dose-dependent decrease in Epo secretion by HepG2 cells ($p \leq 0.05$). In contrast to this, the decrease observed with conditioned medium from LPS-stimulated macrophages that were treated with dexamethasone, was substantially less and differed significantly ($p \leq 0.05$) from that of untreated macrophages cultures (Figure 2.3a). IL-1ß (Figure 2.3b), IL-6 (Figure 2.3c) and TNFα (Figure 2.3d) also resulted in statistically significant ($p \leq 0.05$) dose-dependent reductions in Epo synthesis. Interleukin-1ß was the most potent cytokine with a half-maximal inhibitory concentration of 5 U/ml compared with 70 U/ml for TNFα. IL-6 was the least potent inhibitor of Epo production. A half-maximal inhibitory concentration for this cytokine could not be obtained from the graph, since the highest dose (1000 U/ml) only induced a 44% inhibition in Epo secretion. Neopterin had no effect on Epo synthesis (Figure 2.3e).



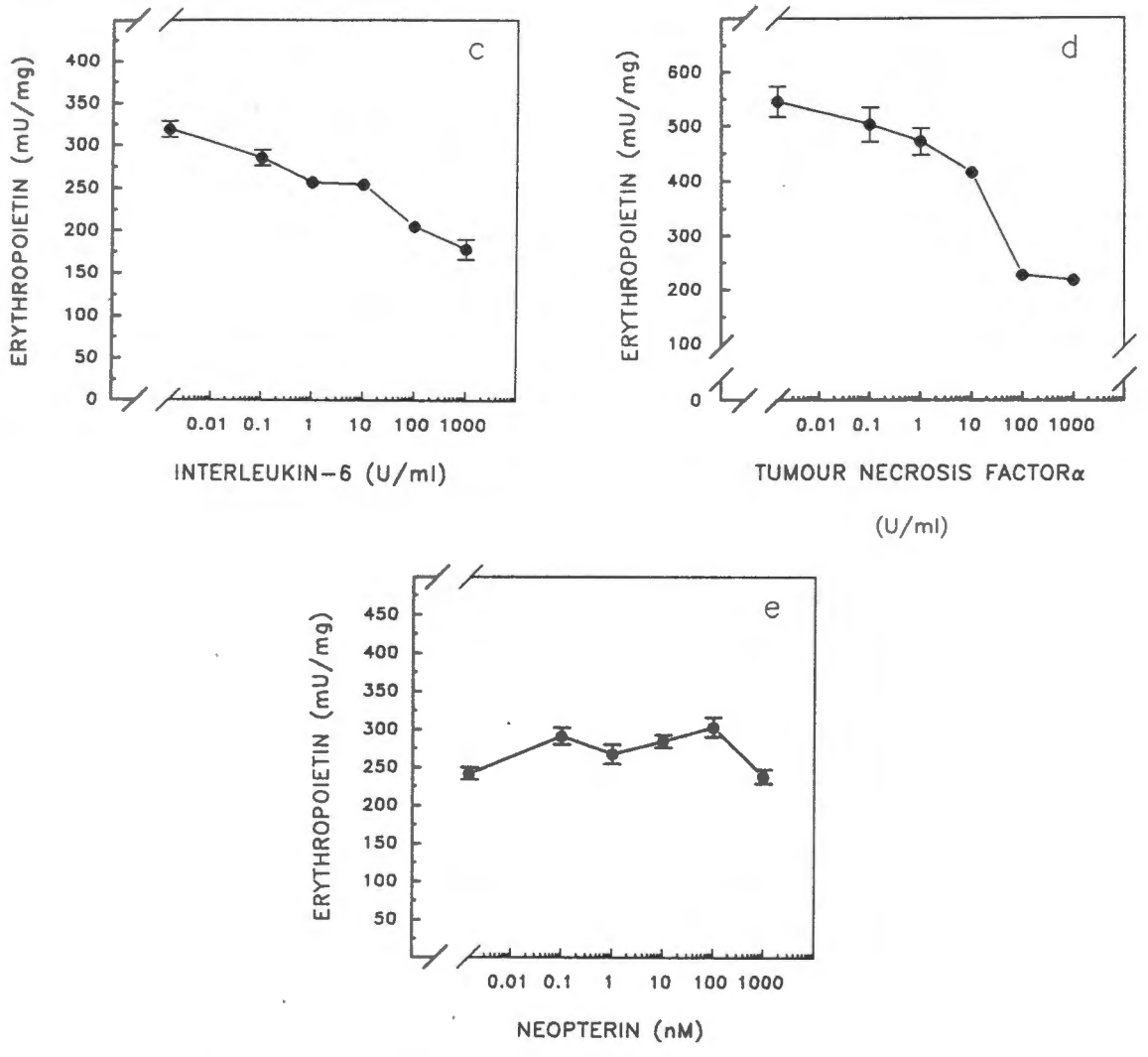


Figure 2.3. Dose-response effects of a) MCM, b) IL-1β, c) IL-6, d) TNFα, and e) neopterin on Epo production by HepG2 cells in culture. All, except neopterin, inhibited the secretion of the hormone by the cells. The inhibitory effect of MCM (○) was partially abrogated by dexamethasone (●). Data are representative of three independent experiments.

2.3.4 CYTOKINE COMBINATION EFFECTS

Interleukin-1ß and TNFα enhanced each other's effects on Epo production by HepG2 cells (Figure 2.4). The inhibition in Epo synthesis obtained with a combination of the two cytokines was significantly more ($p \leq 0.05$) than the reduction observed with each cytokine alone. IL-6 did not augment the inhibitory effects of either IL-1ß or TNFα. The reduction in Epo secretion observed when all three cytokines were simultaneously incubated

with HepG2 cells, was significantly greater ($p \leq 0.05$) than that achieved with IL-6 and TNFα alone ($p \leq 0.05$), but not greater than that of IL-1ß or with a combination of IL-1ß and TNFα .

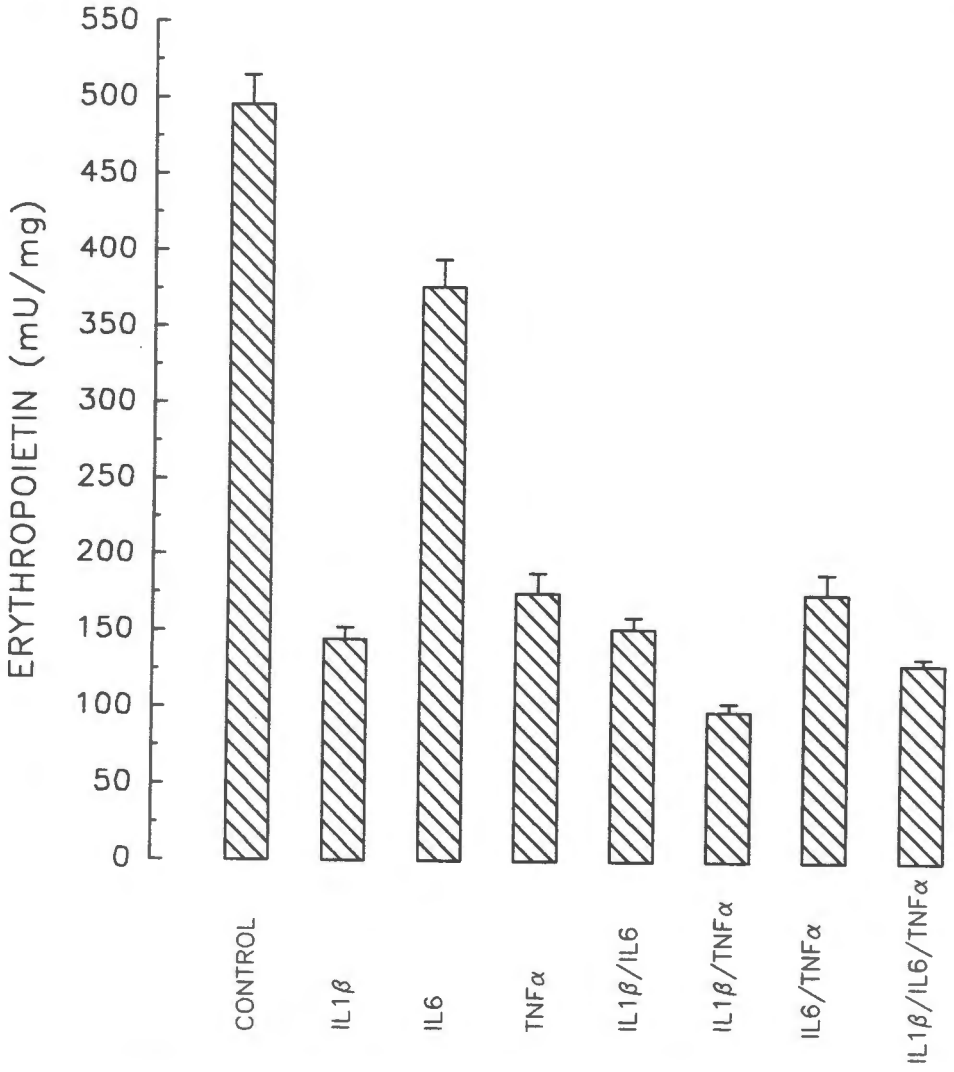


Figure 2.4. Additive effects of IL-1β, IL-6, and TNFα on Epo production by HepG2 cells. Potentiation occurred between IL-1β and TNFα, but not between IL-6 and the other two cytokines. Data are representative of three independent experiments.

Neopterin did not augment the reduction in Epo synthesis obtained with IL-1ß, TNFα, or with all three cytokines used together (Figure 2.5). However, it did effect a significant potentiation in the inhibition observed with IL-6 ($p \leq 0.05$)

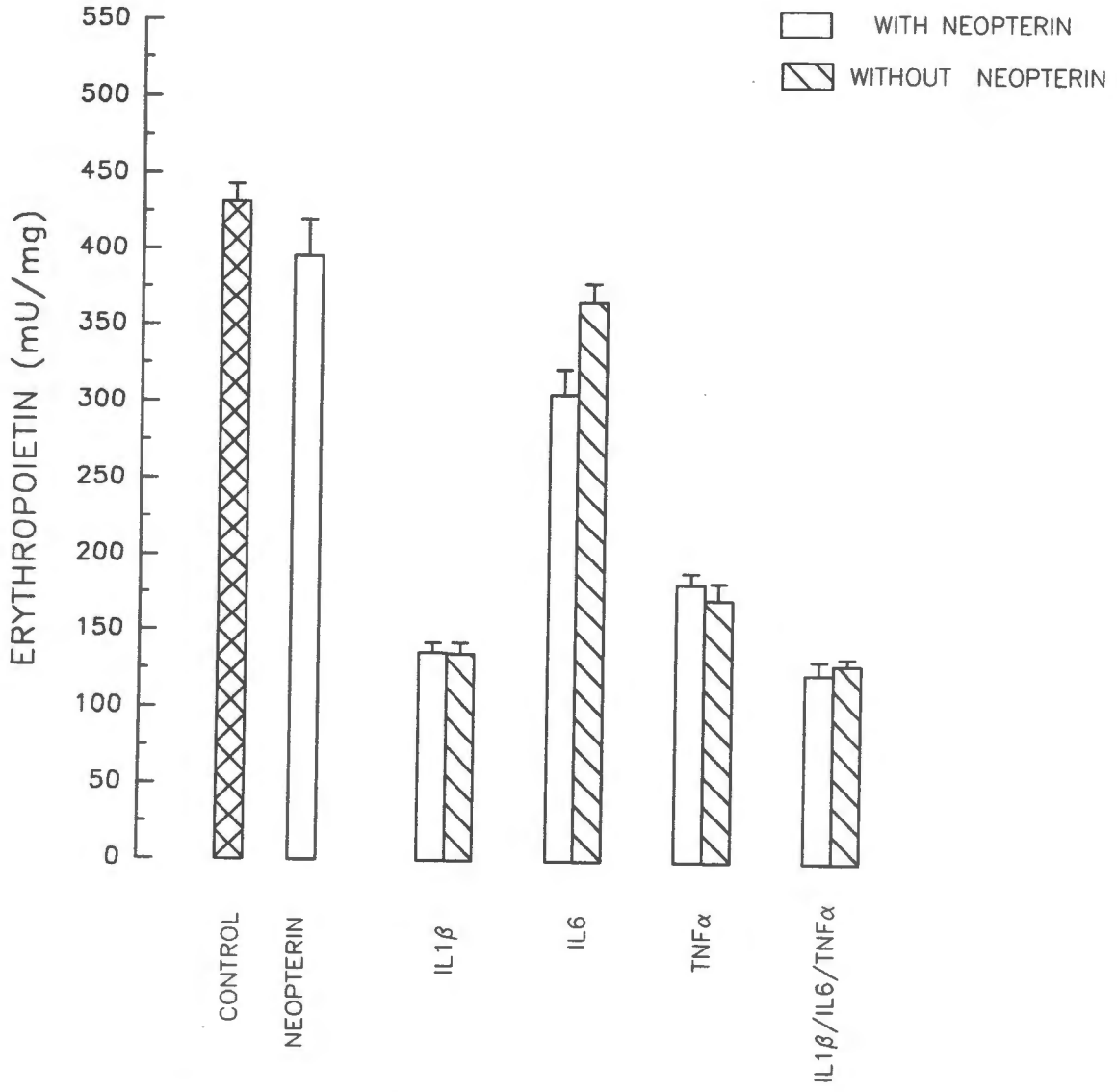


Figure 2.5. The effect of neopterin on IL-1β-, IL-6-, and TNFα-mediated suppression of Epo biosynthesis. Neopterin potentiated the effect of IL-6 only.

All three cytokines, IL-1ß, IL-6, and TNFα, caused a significant enhancement in the inhibition of Epo secretion achieved with conditioned medium from LPS-stimulated macrophages ($p \leq 0.05$). The magnitude of the additive effect on MCM-mediated Epo inhibition was similar for all three cytokines (Figure 2.6). An even greater inhibition of Epo production was observed when the HepG2 cultures were incubated with a mixture of the three cytokines and MCM.

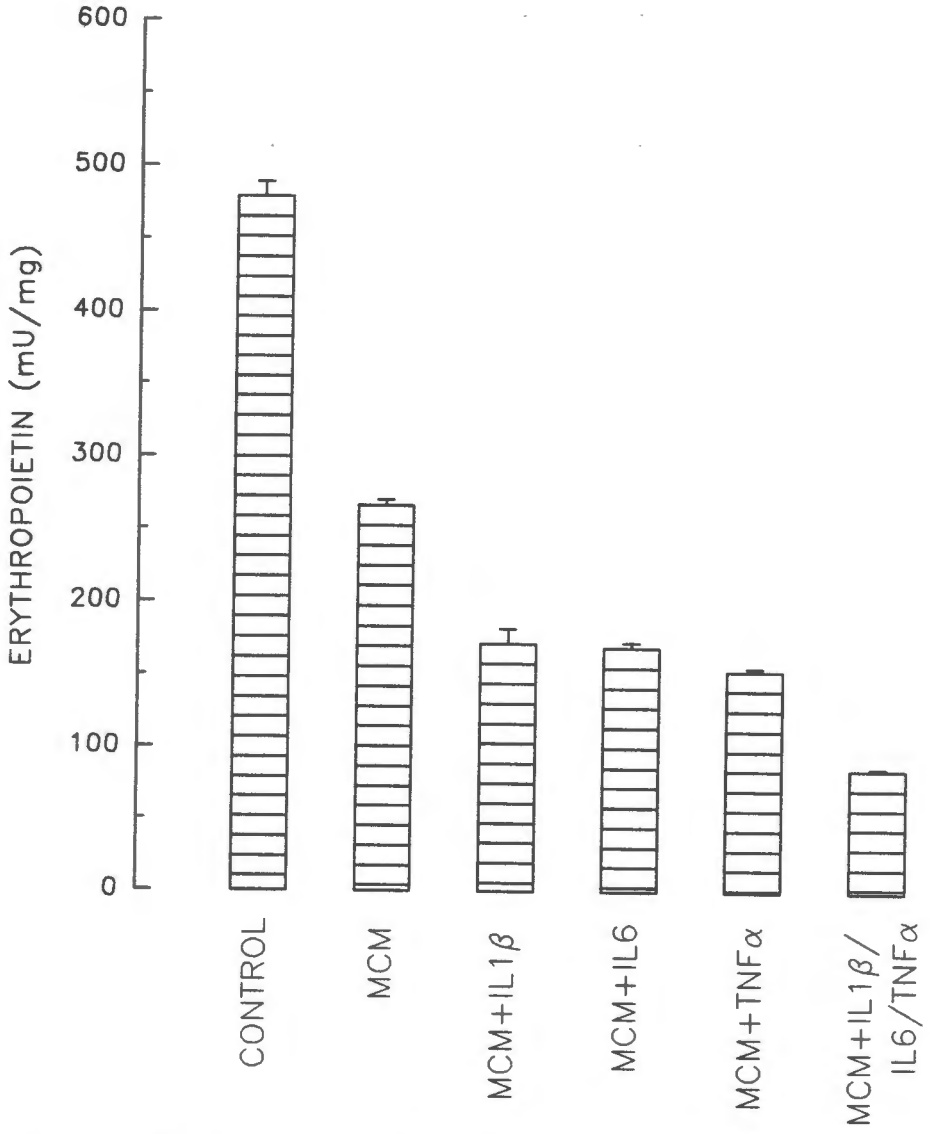


Figure 2.6. The effect of IL-1β, IL-6, and TNFα on the MCM-mediated inhibition of Epo synthesis by HepG2 cells. The response to MCM was enhanced by each cytokine. Greater enhancement was observed when MCM was combined with a mixture of the three cytokines. Data are representative of three independent experiments.

2.4 DISCUSSION

In this study, mediators of the inflammatory response were investigated for suppressive effects on *in vitro* erythropoietin production by cells of the hepatoma cell line, HepG2. HepG2 cells express the Epo gene in a constitutive and regulated manner (Goldberg *et al.*, 1987; Ueno *et al.*, 1989). When these cells are

cultured in a hypoxic atmosphere (1% O_2), they increase their output of Epo. Under normoxic culture conditions (21% O_2), the amount of Epo that they secrete is substantially less. Since the secretion of the hormone by Epo-producing cells in the body is similarly regulated by oxygen tension in the blood, HepG2 cells can serve as an appropriate model for studying Epo synthesis.

None of the test agents, in the concentration ranges used, were found to be toxic to HepG2 cells. Macrophage-conditioned medium (Leu *et al.*, 1991; Van de Loosdrecht *et al.*, 1993) and TNFα (Cairns *et al.*, 1992; Helson *et al.*, 1975) reportedly induce cell-death in certain tumour cell lines. HepG2 cells, derived from a primary hepatoblastoma in a child (Knowles *et al.*, 1980) was, however, insensitive to the toxic effects of either TNFα or MCM.

The growth rate of HepG2 cells was also not significantly affected when cultured in the presence of the test compounds. Although the growth rates were slightly lower, there was no statistical difference between the mean growth rate of control and test cultures. Epo concentration is nevertheless expressed in terms of units per mass of cellular protein (mg), as opposed to units per volume of culture medium (ml), to adjust for this slight variation. A reported decrease in Epo concentration is, therefore, specific and not the result of a cytokine-mediated generalized decrease in protein synthesis (which would manifest as reduced cell growth).

Macrophage-conditioned medium (MCM), obtained from LPS-stimulated mouse peritoneal macrophages, caused a significant ($p \leq 0.05$) and dose-dependent reduction in the amount of Epo secreted by HepG2 cells in culture. This decrease was significantly ($p \leq 0.05$) less in the case of conditioned medium obtained from dexamethasone-treated, LPS-stimulated, macrophages. Glucocorticoids, such as dexamethasone, down-regulate the

expression of such cytokine genes as IL-1 (Lew *et al.*, 1988), IL-6 (Tobler *et al.*, 1992), and TNFα (Beutler *et al.*, 1986). Inhibition of Epo synthesis by MCM could, therefore, have been mediated by these three cytokines.

IL-1ß, IL-6, and TNFα all effected dose-dependent decreases in Epo production. The most potent inhibitor was IL-1ß with a half-maximal inhibitory concentration (IC_{50}) of 5 U/ml, followed by TNFα with an IC_{50} of 70 U/ml. An IC_{50} for IL-6 could not be determined from its dose-response curve since the highest dose (1000 U/ml) only induced a 44% reduction in Epo output. A 25% inhibition in Epo secretion was induced with an IL-6 concentration of 45 U/ml (IC_{25}). For IL-1ß, the IC_{25} was 0.4 U/ml and for TNFα it was 12 U/ml. Interleukin-6 is, therefore, a weak inhibitor of Epo synthesis. Neopterin, unlike the other test compounds, had no effect on Epo secretion.

The cytokines, IL-1ß, IL-6, and TNFα reportedly act synergistically in modulating acute phase protein gene expression (Andus *et al.*, 1988; Helle *et al.*, 1988). IL-1ß and TNFα were found to enhanced each other's effects on Epo synthesis. Their combined effect was significantly greater ($p \leq 0.05$) than the inhibition observed with either cytokine alone. No synergism was, however, observed between IL-6 and the other two cytokines. It has been reported, that IL-6 may counteract the inhibitory effects of IL-1ß and TNFα on Epo production (Faquin *et al*, 1992). In our study, this was found not to be the case. The inhibition obtained with IL-6 and IL-1ß or IL-6 and TNFα was neither greater, nor less, than that achieved with either IL-1ß or TNFα alone.

Neopterin, a macrophage product with no effect on Epo production by HepG2 cells, did not contribute to the Epo inhibitory effects of IL-1ß or TNFα. It did, however, cause a slight, but nevertheless significant ($p \leq 0.05$), potentiation in the

inhibition mediated by IL-6 on Epo synthesis.

Each of the three cytokines, IL-1ß, TNFα, and IL-6 enhanced the inhibition of Epo synthesis obtained with MCM. Greater synergism was seen when all three cytokines were combined with MCM ($p \leq 0.05$). An interesting observation was that the degree of potentiation in the effect of MCM obtained with IL-6 was not less than that achieved with either IL-1ß or TNFα. As mentioned earlier, IL-6 failed to enhance the Epo inhibition obtained with IL-1ß or TNFα. It, therefore, appears that MCM, apart from containing IL-1ß and TNFα, may also contain another factor which can synergize with IL-6 to induce significant inhibition in Epo synthesis. It is unlikely that this unknown factor is neopterin since the degree of synergism observed between neopterin and IL-6 was not as great as that seen with IL-6 and MCM.

This study demonstrates that macrophages, cells prevalent during inflammatory episodes in the body, secrete factors which are potent inhibitors of erythropoietin synthesis. The expression and, hence, secretion of these factors can be modulated by dexamethasone, a glucocorticoid, which almost completely abrogated the Epo inhibitory effect of MCM. The results also suggest that the macrophage factors, which mediate this inhibition, could include IL-1ß, TNFα, and IL-6. IL-1ß and TNFα acted synergistically in exerting inhibition in Epo synthesis. It is likely that another factor, or factors, are secreted by macrophages which can interact with IL-6 to induce substantial reduction in Epo secretion by HepG2 cells in culture.

CHAPTER THREE

MECHANISMS OF CYTOKINE-INDUCED INHIBITION OF ERYTHROPOIETIN SYNTHESIS

3.1 INTRODUCTION

Interleukin-1ß, IL-6, and TNFα, each effected a dose-dependent suppression in the secretion of Epo by HepG2 cells in culture (Chapter 2). These cytokines are known to exert their bioactivities by binding to complementary receptors on the surface of target cells (Sims *et al.*, 1988; Yamasaki *et al.*, 1988; Schall *et al.*, 1990). The receptors are transmembrane cell surface polypeptides that consist of an intracellular domain, concerned with transducing signals to the nucleus, and an extracellular cytokine-binding portion. The association of cytokine with the binding domain of the receptor regulates the activity of the cytoplasmic portion of the receptor molecule, thus delivering a signal which is imparted to the nucleus (Arai *et al.*, 1990; Mixajima *et al.*, 1992). The biochemical nature of the intracellular signalling pathway is virtually unknown. It has, however, been established that protein tyrosine phosphorylation occurs which ultimately leads to modulation of gene transcription via transcription factors (Mixajima *et al.*, 1992). Transcription factors are nuclear proteins that recognize and bind specific regulatory DNA sequences and stimulate or repress transcription (Latchman, 1993).

The inhibited Epo synthesis induced by IL-1ß, IL-6, and TNFα could, therefore, be a transcriptional event in which case there would be a reduction in the concentration of transcribed Epo

mRNA. Alternatively, the inhibition could occur at a post-transcriptional level. Mechanisms exist in cells which regulate the rate of mRNA translation of certain proteins, thereby controlling their average rate of synthesis (Darnell *et al.*, 1990). Translation control is usually effected by substances that can regulate the efficiency of translational initiation. If the translation of Epo mRNA is subject to such a regulatory mechanism, the decreased secretion of the hormone by HepG2 cells may be a consequence of a reduced rate of translation mediated by the three cytokines. In such a case, the level of transcribed Epo mRNA would not be altered.

In order to elucidate the mechanism by which IL-1ß, IL-6, and TNFα inhibited the synthesis of Epo by HepG2 cells, the level of Epo mRNA expression in cells cultured in the presence and absence of an inhibitory concentration of each cytokine was determined.

3.2 MATERIALS AND METHODS

3.2.1 REAGENTS

Human recombinant IL-1ß was a gift from the Biological Response Modifiers Program (Frederick, MD). Interleukin-6, TNFα, *Eco*RI, *Pst*I, lambda and pox DNA markers were purchased from Boehringer-Mannheim (SA). Foetal calf serum was obtained from Highveld Biological (Sandton, SA) and trypsin, phosphate-buffered saline (PBS), and alpha-minimum essential medium (α-MEM) were from Gibco (Grand Island, NY). Agar, Bacto-tryptone and Bacto-yeast extract were from Difco (Detroit, MI). Bovine serum albumin (BSA), transcription buffer (5X), dithiothreitol (DTT), DNase I, RNase ONE and SP6 polymerase were obtained from Promega (Madison, WI). Agarose, ethidium bromide (EtBr), formamide, MOPS (3-[N-morpholino]propanesulphonic acid), sodium acetate, sodium lauryl

sulphate (SLS) and tris(hydroxymethyl)aminomethane(Tris) were bought from Sigma (St Louis, MO). Acrylamide, ammonium persulphate, bisacrylamide (NN'-methylene-bis-acrylamide), TEMED (N,N,N',N'-tetramethylethylenediamine) and urea were obtained from BDH (Poole, England). Sephadex G50 was purchased from Pharmacia (Uppsala, Sweden).

3.2.2 CELLS AND CELL CULTURE

HepG2 cells were obtained from Dr B B Knowles (Wistar Institute, Pennsylvania). The cells were cultured in α-MEM supplemented with 10% FCS (not heat-inactivated) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. (This cell line cannot proliferate in medium that has been fortified with heat-inactivated FCS). Cells were passaged once per week by trypsinization. IW32 cells, a mouse erythroleukemic cell line that constitutively expresses Epo, were a gift from Dr C Lacombe (Inserm, France). The cells were maintained in α-MEM with 5% heat-inactivated (56°C for 30 min) FCS. Cells were passaged twice per week by diluting the culture 1:10 with medium.

3.2.3 PROBES

The rat Epo probe was generously provided by Dr P J Ratcliffe (John Radcliffe Hospital, Oxford). The probe was obtained by amplification of rat genomic DNA using the polymerase chain reaction (PCR). Oligonucleotides complementary to conserved regions in exons IV and V of the published mouse sequence were used as primers. From the amplification products, a *PstI*/*SacI* fragment, containing 132 base pairs (bp) of exon V and approximately 300 bp of the adjoining intron, was constructed and inserted into *p*SP64 (Ratcliffe *et al.*, 1990).

The human Epo cDNA probe, inserted at the *Eco*RI site of the polylinker in *p*SG5, was received from Dr M A Goldberg (Harvard Medical School, Boston, MA).

Upon receipt of the plasmids (*p*SG5 and *p*SP4), they were amplified by introducing them into competent *E. coli* bacteria as described below. This generated a large supply of probe DNA.

3.2.3.1 PREPARATION OF COMPETENT *E. coli* BACTERIA

The method employed was adopted from Sambrook *et al.* (1989). The DK 1.1 strain of *E. coli* was used. To 50 ml of LB medium (Appendix) was added 0.5 ml of an overnight culture of *E. coli*. This was incubated at 37°C in a shaking water bath until the optical density at 600 nm was 0.4. The culture was chilled on ice for 10 min. This was followed by transferring 20 ml of the cell suspension to a 50 ml sterile screw-cap tube (Falcon) and centrifuging it at 3 000 rpm for 10 min at 4°C. The supernatant was discarded and the cell pellet resuspended in 10 ml ice-cold 50 mM $CaCl_2$. This was placed on ice for 60 min. The suspension was re-centrifuged as before, supernatant discarded, and the cells, which were now competent, resuspended in 1 ml ice-cold 50 mM $CaCl_2$. To 200 µl of cell suspension in a sterile Eppendorf tube, was added approximately 50 ng of plasmid DNA. After mixing, the preparation was stored on ice for 30 min. The next step was to heat-shock the cells by transferring them to a water bath at 42°C for 2 min. LB medium (1 ml) was added to each tube, and the cells incubated at 37°C for 60 min. Different volumes (50, 100, and 150 µl) of the transformation mixture and blank were plated on sterile agar plates containing ampicillin (50 µg/ml). A sterile bent-end glass rod was used to spread the cells evenly over the surface of the plates. Plates were incubated inverted at 37°C overnight, and inspected for colony growth the next day.

3.2.3.2 ISOLATION AND PURIFICATION OF PLASMID DNA.

The method followed is a modification of one in Davis *et al.*(1986). A transformed colony was picked from the agar plate prepared above, inoculated into 5 ml of LB medium containing ampicillin (50 µg/ml) and incubated overnight at 37°C in a shaking water bath. The next day 100 ml of LB medium containing the antibiotic was inoculated with 1 ml overnight culture. The culture was incubated at 37°C with vigorous shaking until the optical density at 600 nm was 0.4. To this was added 0.5 ml of stock chloramphenicol solution (34 mg/ml in ethanol) so that the final concentration of chloramphenicol in the culture was 170 µg/ml. Incubation was continued overnight with vigorous shaking.

Bacterial cells were harvested the next day by centrifugation at 2500*g* for 10 min at room temperature. The supernatant was discarded and the pellet suspended in 2.5 ml glucose buffer (Appendix). To this was added 750 µl freshly prepared lysozyme solution (8 mg/ml in glucose buffer). The mixture was allowed to stand at room temperature for 5 min and then transferred to a sterile 30 ml corex tube. Addition of 6 ml of freshly prepared 0.2 M NaOH solution containing 1% SDS followed. It was mixed and placed on ice for 5 min. To this was added 4.5 ml ice-cold potassium acetate solution (Appendix) followed again by mixing with a vortex, and placing it back on ice for a further 10 min. The mixture was centrifuged at 12 000*g* for 10 min at 4°C. The supernatant was carefully transferred to another corex tube, mixed with 0.6 volumes of isopropanol, and placed in a -20°C freezer for 15 min to precipitate the DNA. The DNA was recovered by centrifugation at 12 000*g* for 15 min at 4°C. The supernatant was discarded, the DNA dissolved in 400 µl TE buffer (Appendix), and transferred to an Eppendorf tube. The DNA solution was further purified by extracting it first with one volume of a mixture of phenol and chloroform (1:1) and then with one volume of chloroform alone. After the two phases had separated, the

upper aqueous phase was transferred to a new Eppendorf tube, and mixed with a tenth volume of 3 M sodium acetate solution (pH 7.4) and two and a half volumes of absolute ethanol. The mixture was placed at -20°C for 1 h, centrifuged at 12 000*g* for 10 min at 4°C, and the supernatant discarded. The pellet was washed with 70% ethanol, dried in a vacuum chamber, and redissolved in 200 µl TE buffer. The concentration of the DNA was determined by measuring the absorbance at 260 nm of a 1:500 diluted solution, and the purity assessed by calculating the ratio of the absorbance values at 260 and 280 nm.

3.2.3.3 PROBE ISOLATION

The rat Epo probe was isolated by digesting the *p*SP64 plasmid with the restriction enzymes *Pst*I and *Eco*RI. The *p*SG5 plasmid preparation was digested with *Eco*RI to yield the human Epo cDNA probe. All enzyme reactions were carried out in appropriately buffered solutions in a 37°C water bath for 2 h. The digests were subjected to electrophoresis at 80 volts through 1% agarose gels with 1X TBE (Appendix) as running buffer. After electrophoresis, the gels were stained in dilute ethidium bromide (0.5 µg/ml) solution.

Probe bands were cut out with a sharp razor blade. The DNA was eluted from the gel pieces by placing them on a bed of sterile glass wool inside a small Eppendorf tube (0.5 ml) with a hole punctured through the bottom. The gel pieces were frozen in liquid nitrogen, the tube fitted inside a bigger Eppendorf tube (1.5 ml), and spun at 13 000 rpm for 20 min at room temperature. The supernatants carrying the probes flowed through the hole in the bottom of the small tube into the larger one.

The DNA solutions were extracted once with one volume of a mixture of equal parts of phenol and chloroform and then with one

volume of chloroform alone. Probes were precipitated with 0.3 M sodium acetate and 2.5 volumes of absolute ethanol, and placed at -20°C for 1 h. The pellets were spun down at 13 000 rpm for 15 min at 4°C, washed in 70% ethanol, vacuum-dried, and then redissolved in TE to give 50 to 150 ng/µl solutions in which they were stored at -20°C.

3.2.3.4 RADIOACTIVE LABELLING OF PROBE

Labelling of probes was performed with the Multiprime DNA labelling system (Amersham, RPN 1600Y). Probes were labelled with [α-^{32}P]dCTP (Amersham) to give 1x10^{6} cpm per ml of hybridisation solution. Labelling proceeded as follows: 50 ng of probe DNA in 5 µl TE was denatured in a 95°C water bath for 5 min and then transferred directly onto ice. To this was added 10 µl of concentrated buffer solution containing hexanucleotides of random sequence which bind to the denatured probe DNA, plus dATP, dTTP, and dGTP. This was followed by the addition of 5 µl of solution which contained the Klenow fragment of *E. coli* DNA polymerase I, and 5 µl of [α-^{32}P] dCTP. The reaction mixture was made up to 50 µl with sterile distilled water, mixed with a vortex and left overnight at room temperature. The next morning, the reaction was stopped by adding 1 µl 10% SDS and 20 µg denatured salmon sperm (ss) DNA. Labelled probe was separated from unincorporated nucleotides by size exclusion chromatography on a Sephadex G50 (fine) column using TE as elution buffer. The radioactivity was measured by counting 10 µl of the 1 ml eluate containing the labelled probe in 5 ml toluene scintillant with a scintillation counter (Packard Tri-carb 4530). The labelled probe was denatured again by boiling for 5 min and immediately transferred to ice. It was then ready for hybridisation.

3.2.4 RNA EXTRACTION

Glassware was washed in chromic acid solution (Appendix), rinsed several times with distilled water and dried in an oven (100°C). The clean glassware was then siliconized by dipping it in a 1 % dichloromethylsilane solution and baking it at 120°C for 1 h. After baking, the glassware was rinsed with distilled water and treated with 0.01% diethylpyrocarbonate (DEPC). All aqueous solutions, except those containing Tris, were also treated with 0.01% DEPC. DEPC inactivates ribonucleases. These enzymes, which degrade RNA, are very stable and found everywhere (glass surfaces, bench tops, hands, etc.). After DEPC treatment (which was carried out at room temperature for 3 to 5 h), solutions and glassware were autoclaved for 20 min at 121°C to remove the volatile DEPC and for sterilization. Electrophoresis tanks were cleaned with detergent solution, rinsed in water, and then filled with a solution of 10% H_2O_2. After 10 min at room temperature, the tanks were rinsed in DEPC-treated distilled water and left upside down on clean absorbent paper to dry. Gloves were worn throughout all experimental procedures.

3.2.4.1 EXTRACTION OF TOTAL RNA

IW32 cells, which grow in suspension, were collected by centrifugation at 2 000 rpm for 5 min at room temperature, and washed three times by resuspension in ice-cold PBS lacking calcium and magnesium ions. HepG2 cells, which grow in monolayers, were trypsinized first, then centrifuged and washed as above. After estimating the volume of packed cells, the cells were resuspended in 10 volumes of RNA extraction buffer (Appendix). The cell suspensions were mixed on a vortex, placed on ice for 5 min, mixed again and transferred to sterile Eppendorf tubes. The preparations were centrifuge in a Biofuge A (Heraeus) at 13 000 rpm for 2 min and supernatants transferred

to 15 ml sterile corex tubes. To these were added one volume of proteinase digestion buffer (Appendix). After mixing on a vortex, proteinase K was added to a final concentration of 50 µg/ml. The solutions were mixed again and then incubated for 30 min in a 37°C water bath. Proteins were removed by extracting twice with an equal volume of phenol (containing 0.1% hydroxyquinoline), then once with a 1:1 mixture of phenol and chloroform/amyl alcohol solution (25:1) and then finally once with chloroform/amyl alcohol alone. The aqueous layer was transferred to a sterile plastic tube to which was added 0.3 M sodium acetate, pH 7.5, and 2.5 volumes absolute ethanol. Total RNA was allowed to precipitate at -20°C overnight. The precipitated RNA was centrifuged at 12 000 rpm for 10 min at 4°C. The supernatant was discarded, the pellet washed with 70% ethanol, and then vacuum-dried. The pellet of RNA was redissolved in 200 µl TE (pH 7.6) and its concentration determined spectrophotometrically at 260 nm (Shimadzu, uv-120-02). The concentration was calculated based on an OD_{260} reading of 1 for a RNA solution of 40 µg/ml. The RNA solution was stored at -20°C after addition of 500 µl absolute ethanol.

3.2.4.2 ISOLATION OF POLY(A)$^+$ RNA

Messenger RNA was isolated from other forms of RNA (tRNAs and rRNAs) by affinity chromatography on an oligo(dT)-cellulose column. A slurry of the oligo(dT)-cellulose was prepared in 0.1 N NaOH, and the column packed in a sterile, siliconized pasteur pipette plugged with sterile, DEPC-treated glass wool. The column was washed with 3 column volumes of sterile, DEPC-treated water, and then with sterile 1X column loading buffer (Appendix) until the pH of the effluent was less than 8. The total RNA sample, dissolved in sterile water, was heated to 65°C for 5 min, rapidly cooled to room temperature, mixed with 2X column loading buffer (Appendix), and applied to the column. Once all the RNA

solution had entered the column, one column volume of 1X column loading buffer was added. Eluate was collected continuously in a sterile tube. When all the solution eluted from the column, the eluate was heated to 65°C for 5 min and reapplied to the column.

The column was washed with 5 to 10 column volumes of 1X column loading buffer while collecting 1 ml fractions in sterile Eppendorf tubes. The absorbance of each fraction was measured at 260 nm. When the fractions showed no absorbance, indicating the absence of nonpolyadenylated RNA, poly $(A)^+$ RNA was eluted from the oligo(dT)- cellulose with 2 to 3 column volumes of sterile, RNase-free elution buffer (Appendix). The absorbance of the eluate, collected in fractions, was measured at 260 nm, followed by pooling of those fractions which contained RNA. To the pooled fractions were added sodium acetate (pH 5.2), to a final concentration of 0.3 M, and 2.5 volumes of ice-cold absolute ethanol. After mixing on a vortex, the preparation was stored at -20°C overnight. The next day, the poly$(A)^+$ RNA was recovered by centrifugation at 10 000*g* for 15 min at 4°C. The supernatant was discarded, the pellet washed with 70% ethanol, and then vacuum-dried. Finally, the RNA was redissolved in a 100 µl sterile, DEPC-treated water and its concentration determined spectrophotometrically.

3.2.5 RNA ANALYSIS

3.2.5.1 FORMALDEHYDE GEL ELECTROPHORESIS OF RNA

RNA was electrophoresed on a 1.1% agarose gel containing 2.2 M formaldehyde. The gel was prepared by mixing 170 mg agarose with 1.5 ml 10X MOPS buffer (Appendix) and 12.75 ml sterile DEPC-treated deionized water. After heating to dissolve the agarose, the mixture was cooled to 40°C under running tap water.

Formaldehyde (0.75 ml) was then added and the mixture poured onto a 5 x 7.5 cm perspex plate on which was positioned a perspex comb about 1 cm from the narrow top edge. The gel was allowed to solidify at room temperature after which the comb was gently removed. RNA samples in sterile, DEPC treated water, were mixed with the same volume of RNA loading dye mixture (Appendix) and loaded into the wells created by the perspex comb. The samples were electrophoresed at 60 volts for 2 h using 1X MOPS as gel running buffer. Standards, consisting of *E. coli* rRNA, were also included. After electrophoresis, the gel was transferred to a dilute solution of ethidium bromide in 1X MOPS. The gel was left in this solution for 5 to 7 min to allow the EtBr to stain the RNA. After staining, the gel was inspected on a transilluminator (Spectroline, model TS-302) and photographed.

3.2.5.2 NORTHERN TRANSFER

After electrophoresis, the formaldehyde gel was washed in 0.1 N NaOH for 20 min to ensure complete denaturation of RNA. This was followed by washing the gel in DEPC-treated water for 20 min and then equilibrating it twice in 10X SSC (Appendix), also for 20 min. The gel was mounted on a filter paper wick which dipped into a reservoir of 10X SSC (transfer buffer). The Hybond-N nylon hybridisation membrane (Amersham), cut to the same size as the gel, was sandwiched between the gel and three pieces of filter paper (Whatman) of the same size. The filter paper in contact with the nylon membrane was first soaked in 2X SSC. On top of this was stacked paper towels (5 to 8 cm high) which served to draw the transfer buffer through the gel by capillary action. The whole assembly was compressed by a 500 g weight. Blotting was carried out overnight (8 to 12 h). The paper towels and filter paper were discarded and the membrane inspected with the transilluminator. The positions of the *E. coli* ribosomal RNA (16S and 23S), which were still stained with EtBr, were marked by

making holes in the membrane with a sharp needle. The nylon membrane was placed between two sheets of Whatman filter paper and baked for 2 h at 80°C. Gels were also inspected to check the efficiency of transfer.

3.2.5.3 HYBRIDISATION

The baked membrane was prewetted in 6X SSC, placed on top of a nylon mesh which prevented the membrane from overlapping, and both mesh and membrane were then rolled into a spiral and placed inside a leak-proof hybridisation bottle. Prehybridisation solution (Appendix), containing denatured ssDNA, was added to the bottle which was then sealed and fitted in the rotisserie unit of the hybridisation oven (Hybaid). The membrane was prehybridised at 42°C for 3 to 4 h. Denatured labelled probe was added directly to the prehybridisation solution to give a final concentration of $1x10^6$ cpm/ml. Hybridisation was then carried out at 42°C overnight.

After hybridisation, the Northern blots were washed as follows:

2X SSC, 0.5% SDS for 10 min at room temperature,
2X SSC, 0.1% SDS for 15 min at room temperature, and
0.1X SSC, 0.1% SDS for 30 min at 65°C.

3.2.5.4 AUTORADIOGRAPHY

After the final wash, the blot was wrapped in Saran Wrap and taken to a darkroom where it was placed on a X-ray film (Cronex-4) between two intensifying screens in a light-proof cassette. Exposure was done at -70°C for 7 to 10 days. The film was developed in the darkroom as follows: 5 min in X-ray developer followed by 1 min in a water bath, 5 min in fixer solution and,

finally, 15 min in running water. All the solutions were at room temperature. The film was eventually air dried and visually inspected for radioactive signals.

3.2.5.5 STRIPPING OF BLOTS

Northern blots were stripped for rehybridisation using a method of Davis *et al.*(1986). Stripping was accomplished by placing the blots in a 1 % glycerol solution for 2 min at 80°C, followed by 4 to 5 washes in DEPC-treated water at room temperature. The blots were then exposed for 5 days to assess the efficiency of the stripping procedure.

3.2.5.6 RIBONUCLEASE PROTECTION ASSAY

Ribonuclease (RNase) protection is an extremely sensitive technique for quantification of specific mRNAs in solution (Sambrook *et al.*, 1989). It is 20 times more sensitive than Northern blots and is, therefore, particularly useful for the detection of low abundance mRNA species. To perform protection assays, high specific activity ^{32}P-labelled RNA probes (riboprobes) complementary to the mRNAs of interest, are used in solution hybridisations. Unhybridised material is removed by digestion with RNase. The protected fragment is analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography.

The riboprobe was synthesized at room temperature by adding the following reagents to an Eppendorf tube in the sequence given:

5 µl [α-^{32}P] UTP (3 000 Ci/mmol, 10 Ci/ml)
4 µl 5X transcription buffer (Appendix)
2 µl 100 mM dithiothreitol (DTT)

0.5 µl RNasin
1 µl GTP (10 mM)
1 µl CTP (10 mM)
1 µl ATP (10 mM)
0.5 µl UTP (0.4 mM)
2 µl BSA (1 mg/ml)
2 µl (1 µg) *Eco*RI-linearized *p*SP64 plasmid DNA with Epo probe.
1 µl DNA-dependent Sp6 RNA polymerase

The reagents were mixed by gently tapping the tube and the mixture was incubated in a 40°C water bath for 2 h. To this was added 0.5 µl RNasin and 1 µl of RNase-free DNase I (1 mg/ml). After further incubation for 15 min at 37°C, 30 µl of column buffer (Appendix) was added and the mixture chromatographed through a Sephadex G50 column. The front peak of radioactivity was collected and the eluate divided into 400 µl aliquots. To each was added 40 µl of 3 M sodium acetate, 10 µg tRNA and 2.5 volumes of ice-cold absolute ethanol. After mixing, the riboprobe solution was placed inside a lead container and stored in a freezer at -20°C overnight.

The riboprobe was collected by centrifugation at 12 000*g* for 15 min at 4°C, washed with 70 % ethanol and resuspended in 100 µl DEPC-treated water. The radioactivity was quantified by dissolving 2 µl of riboprobe solution in 10 ml toluene scintillant and measuring it with a ß-counter.

The hybridisation reactions were started by coprecipitating individual RNA samples and riboprobe (200 000 cpm) with 0.3 M sodium acetate and 2.5 volumes absolute ethanol in 1.5 ml Eppendorf tubes. After centrifugation at 12 000*g* for 15 min at 4°C, the pellets were rinsed with ice-cold 70% ethanol, spun briefly in a microcentrifuge, and dried in a vacuum chamber. The pellets were resuspended in 30 µl hybridisation buffer

(Appendix). Samples were heated at 85°C for 5 min to denature the RNA and then incubated at 47°C for 12 to 16 h to allow hybridisation to occur. Two aliquots of 50 µg tRNA were included as controls; one was treated with RNase and the other was left untreated.

After overnight incubation, 300 µl of RNase digestion buffer (Appendix) and 10 units of RNase ONE were added. A further incubation at 37°C for 60 min followed. Digestion was stopped by adding, to each sample (except tRNA control without RNase), 3.3 µl of 10% SDS and 20 µg carrier tRNA. After mixing with a vortex, 700 µl of ice-cold absolute ethanol was added and the samples chilled at -70°C for 15 min. RNA samples were pelleted by centrifugation at 12 000*g* for 15 min, washed with 0.2 ml 70% ethanol, and vacuum-dried. Pellets were dissolved in 2 µl DEPC-treated water and 4 µl formamide loading dye (Appendix). After heating at 95°C for 5 min, the samples were resolved by electrophoresis at a 1000 volts on a 6% polyacrylamide/7 M urea gel (Appendix). The protected fragments were detected by autoradiography and quantified with a densitometer (Hoefer GS 300).

3.3 RESULTS

3.3.1 PROBE ISOLATION

The 432 bp rat genomic Epo probe was isolated from the *p*SP64 vector by digestion with the restriction endonucleases, *Eco*RI and *Pst*I. A diagnostic gel with the digests was run showing the plasmid vector and excised probe fragment (Figure 3.1a). The *Eco*RI linearized plasmid containing the probe is also shown. A bulk isolation of the fragment of interest was then carried out (Figure 3.1b). A thin section of gel containing the probe was

sliced out and the probe recovered by spin elution.

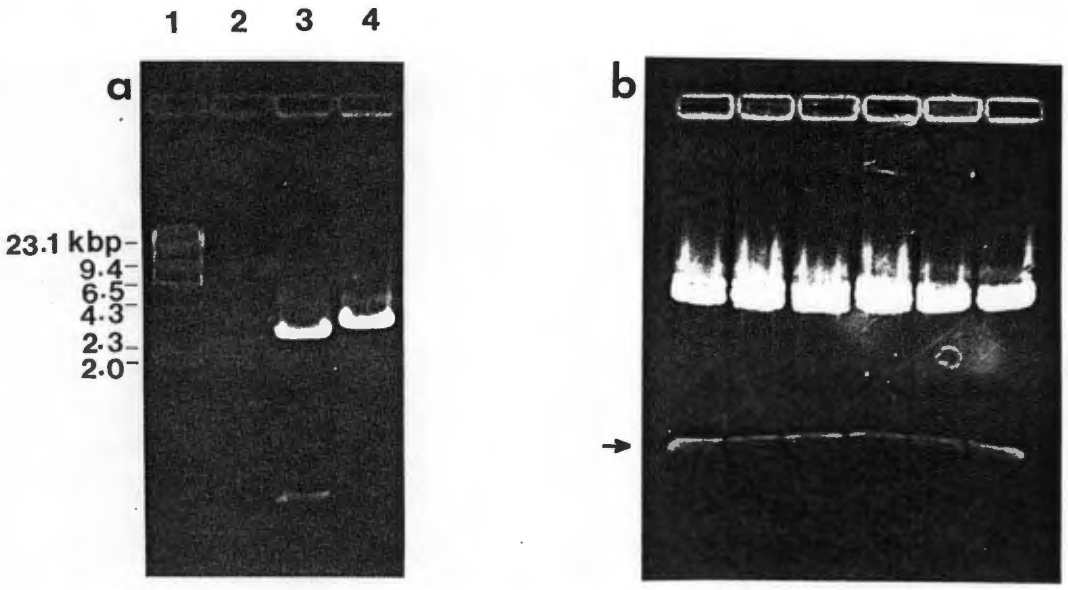


Figure 3.1. Isolation of the 450 bp rat Epo probe. a) Lane 1 contains λ/*Hin*d III DNA markers. Lane 3 shows the probe fragment after digestion of *p*SP64 vector with *Eco*RI and *Pst*I. Lane 4 contains the *p*SP64 vector with the 450 bp Epo insert after linearization with *Eco*RI. b) Bulk isolation of the probe (→) after large-scale digestion of *p*SP64 with *Eco*RI and *Pst*I.

Figure 3.2a shows the human Epo cDNA probe isolated from the *p*SG5 vector after digestion with *Eco*RI. A bulk digestion and isolation of this probe was also carried out (Figure 3.2b).

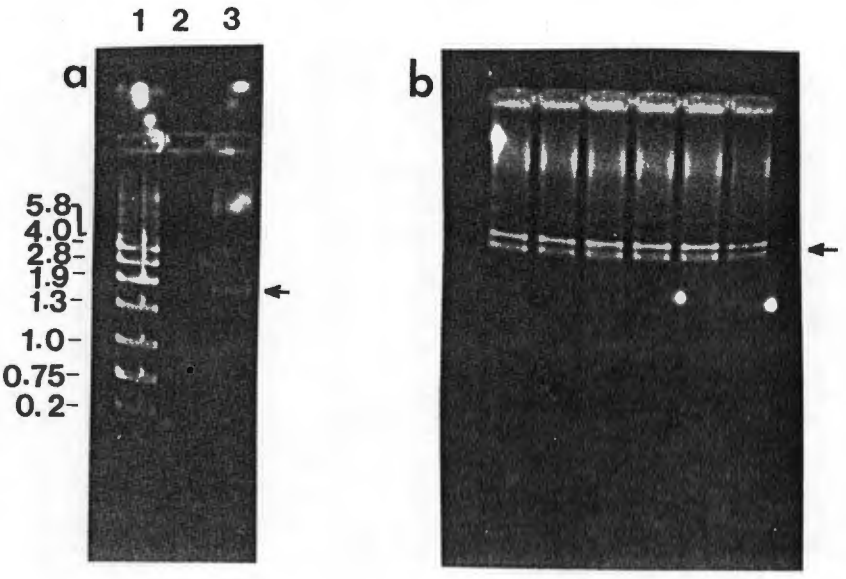


Figure 3.2. Isolation of the human cDNA probe. a) Lane 1 contains the pox/*Eco*RI DNA marker. Lane 3 shows the probe (→) after digestion of the *p*SG5 vector, which contained the probe, with *Eco*RI. b) Mass isolation of the Epo cDNA probe after bulk digestion of the vector with *Eco*RI. The gel containing the fragment of interest was carefully excised and the probe isolated by spin elution.

3.3.2 RNA ISOLATION AND EVALUATION

Total cellular RNA was isolated from IW32 and HepG2 cells by the phenol-chloroform method (Sambrook *et al.*, 1989), and evaluated on a formaldehyde-agarose gel. Figure 3.3a shows the RNA isolated from three separate cultures of IW32 cells. Both 18S and 28S ribosomal RNA (rRNA) bands are distinct, indicating successful extraction. RNA was also successfully extracted from HepG2 cell cultures (Figure 3.3b). The prominent 18S and 28S rRNA bands suggest little or no degradation.

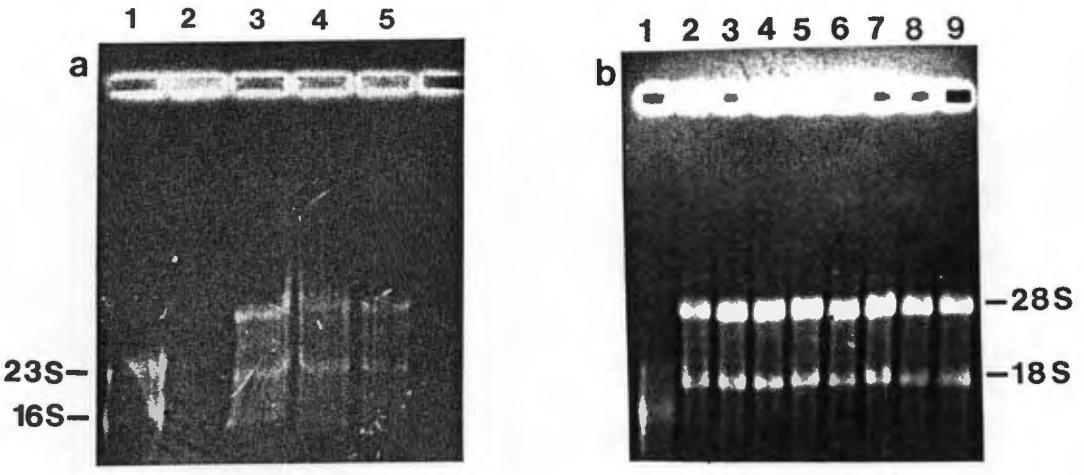


Figure 3.3. Formaldehyde-agarose gels of RNA. a) IW32 RNA after phenol-chloroform extraction (lanes 3 to 5). The 18S and 28S rRNA bands are distinct indicating that the RNA did not degrade during isolation. Lane 1 contains *E. coli* RNA as markers. b) HepG2 RNA in lanes 2 to 9 showing clear rRNA bands.

3.3.3 NORTHERN BLOTS

a) HYBRIDISATION WITH THE RAT EPO PROBE

Northern blot analysis of RNA, extracted from IW32 and HepG2 cells and probed with the *PstI/SacI* fragment of the rat genomic Epo probe, demonstrated an Epo mRNA species only in the IW32 RNA sample. The size of Epo mRNA is about 1.8 kb (Lacombe *et al.*, 1987) which is also the approximate size of mammalian 18S rRNA. The two will, therefore, run together on an agarose gel. The autoradiograph in Figure 3.4a shows an intense band at the 18S position of Lane 1 and a less intense band at the 28S position. In lane 2, which contains the HepG2 RNA sample, the 28S band is more intense than the 18S band. In the photograph of the EtBr-stained gel taken prior to hybridisation (Figure 3.4b) the bands at the 28S position of bath lane 1 (IW32) and lane 2 (HepG2) were more prominent than the 18S bands. If the hybridised signals in lanes 1 and 2 of the autoradiograph represented only

nonspecific binding of the probe to rRNA, then the signals at the 28S position in both lanes should have been more intense than the signals at the 18S position. The higher intensity of the 18S signal in lane 1, compared to the 28S signal, suggests that in addition to nonspecific binding to 18S rRNA the probe also hybridised to a specific Epo transcript. The low intensity signal at the 18S position in lane 2 (HepG2 RNA) of the autoradiograph seems to suggest that the probe is either too specific for rat Epo mRNA and, hence, cannot hybridise to human Epo mRNA or that HepG2 cellular RNA contains much less Epo mRNA than the same amount of IW32 cellular RNA. Attempts at reducing nonspecific binding by increasing the stringency of the washing step resulted in the loss of the hybridised signal. This Northern blot represents the best result of at least eight attempts.

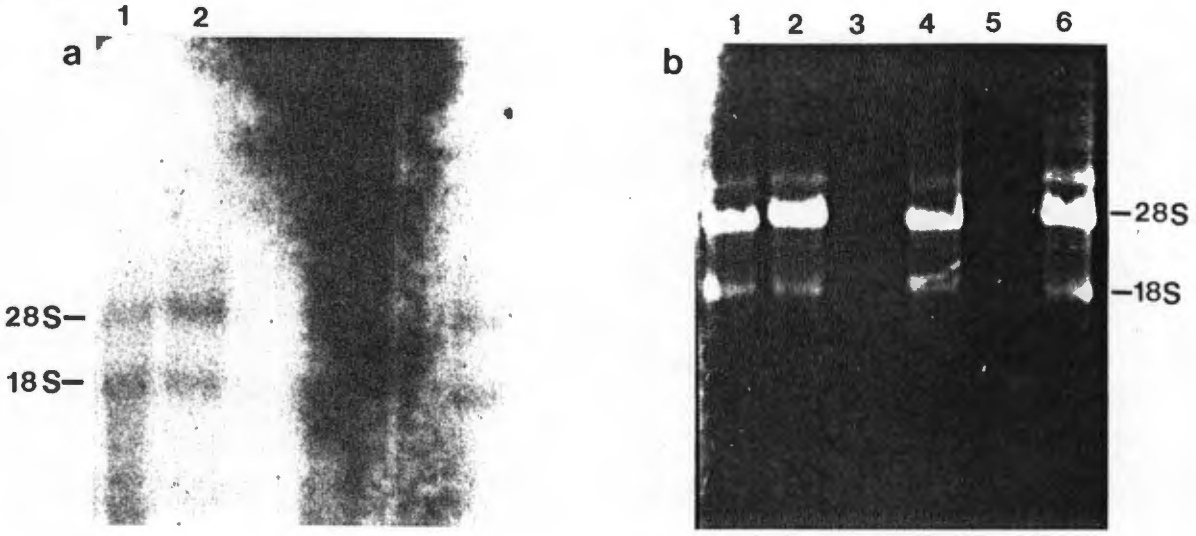


Figure 3.4. Northern blot analysis of IW32 and HepG2 cellular RNA with the rat Epo probe. a) Autoradiograph of IW32 RNA (lane 1) and HepG2 RNA (lane 2). The signal at the 18S position is more intense than the one at 28S in lane 1. In lane 2 the 28S signal is more intense. Epo mRNA migrates with 18S rRNA. The dense signal at 18S in lane 1 could represent nonspecific binding of probe to rRNA and specific hybridisation to Epo mRNA. b) EtBr-stained gel of IW32 (lanes 1 and 4) and HepG2 (lanes 2 and 6) RNA prior to hybridisation. Note how intense the 28S bands are in all the lanes.

b) HYBRIDISATION WITH HUMAN EPO cDNA PROBE

Northern analysis of HepG2 RNA (lane 1) and IW32 RNA (lane 2) with the human Epo cDNA probe showed hybridisation of the probe to mRNA species in the IW32 sample, but not in the HepG2 sample (Figure 3.5). IW32 cells possess two alleles of the Epo gene. The one allele is normal whereas the other is rearranged and amplified (Goldwasser *et al.*, 1987; Lacombe *et al.*, 1987). The two signals in lane 2 may represent hybridisation to transcripts of both alleles, one of which is much shorter than the other. Alternatively, the smaller signal at the bottom could represent hybridisation of the probe to degraded mRNA. What is important to note, however, is that the human Epo cDNA probe failed to hybridise to Epo mRNA in the RNA sample isolated from the human cell line, HepG2.

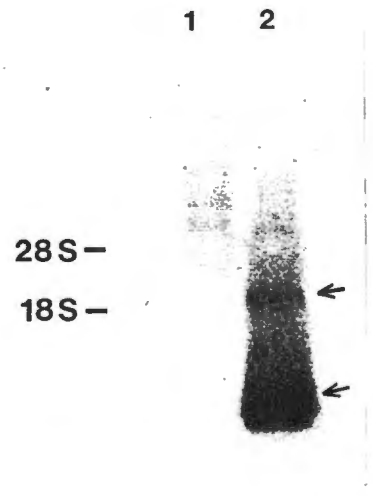


Figure 3.5. Northern analysis of HepG2 and IW32 RNA with the human Epo cDNA probe. Lane 1 represents probe hybridisation to HepG2 RNA. A definite signal is lacking. Lane 2 represents hybridisation to the IW32 RNA sample. Two distinct hybridisation signals are present. This may indicate annealing to two transcripts or the bottom signal may be due to hybridisation to degraded Epo mRNA.

Figure 3.6 is an autoradiograph of a Northern blot of cellular RNA of IW32 and HepG2 cells as well as of poly(A)$^+$ RNA isolated

from total RNA of the two cell lines. The blot was probed with labelled human Epo cDNA. Hybridisation occurred to Epo mRNA in lane 4 which contained IW32 cellular RNA. Lane 3, which contained IW32 poly(A)$^+$RNA, had no definite signal. Lane 2 which was loaded with HepG2 cellular RNA had two hybridisation signals which were located at the 18S and 28S positions. These signals probably represent nonspecific hybridisation to the two rRNA species. Lane 1 was loaded with 20 µg poly(A)$^+$-enriched HepG2 RNA. Hybridisation of a probe to poly(A)$^+$RNA sometimes appear as a smear down the lane. The signal may, therefore, represent hybridisation to Epo mRNA. The reason why no clear signal was detected in the IW32 poly(A)$^+$RNA sample could be due to degradation of the sample.

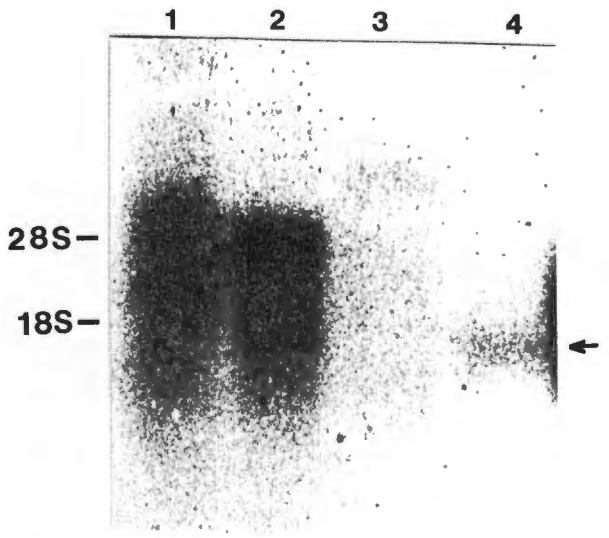


Figure 3.6. Northern blot analysis of total and poly(A)$^+$ RNA isolated from IW32 and HepG2 cultures. Hybridisation occurred to the total IW32 RNA sample (lane 4) but not to the poly(A)$^+$ RNA sample (lane 3), probably because of sample degradation. Lane 2 contained total HepG2 RNA. The two signals in this lane represent nonspecific hybridisation to 18S and 28S rRNA. Lane 1 contained 20 µg of poly(A)$^+$ HepG2 RNA. A smear is usually indicative of hybridisation to a specific mRNA species in a large amount of enriched poly(A)$^+$ RNA.

3.3.4 RNase PROTECTION

Ribonuclease protection analysis of 10-fold different amounts of IW32 cellular RNA was carried out using the ^{32}P-labelled riboprobe synthesised from the *PstI/SacI* fragment of the rat genomic Epo gene. A protected fragment of 132 bp was observed only with the 50 µg RNA sample (Figure 3.7, lane 8). Hybridisation reactions, in which lower amounts of RNA were used, did not yield a protected fragment (lanes 4 to 7).

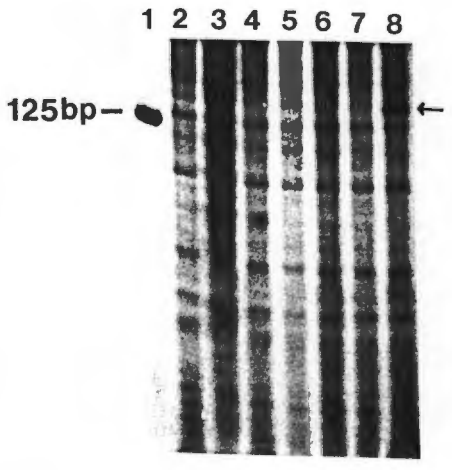


Figure 3.7. Autoradiograph of a protection assay of varying amounts of IW32 RNA probed with the 132 bp rat Epo riboprobe. Only the 50 µg sample (lane 8) yielded a signal indicative of the protected fragment. Lane 1: markers, Lane 2: tRNA with RNase, Lane 3: tRNA without RNase, Lane 4: 0.005 µg IW32 RNA, Lane 5: 0.05 µg, Lane 6: 0.5 µg, Lane 7: 5 µg and Lane 8: 50 µg RNA.

Protection assays with HepG2 RNA also yielded protected fragments of approximately 132 bp in size (see arrow in Figure

3.8a). Densitometric analysis of these fragments showed that the RNA samples isolated from cells treated with IL-1ß and TNFα contained a lower concentration of Epo mRNA than the control sample obtained from untreated cells (Figure 3.8b). The density of the hybridisation signal generated by the RNA sample, extracted from cells treated with IL-6, was not different from that of the control sample. This suggest that IL-6, unlike IL-1ß and TNFα, did not affect Epo mRNA transcription.

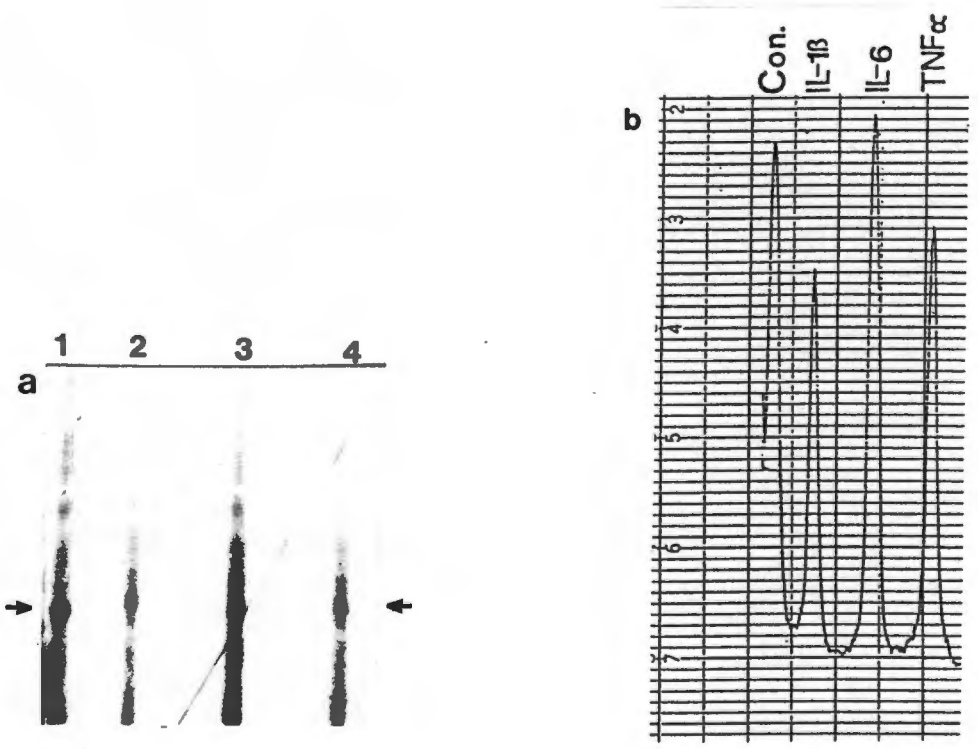


Figure 3.8. Protection analysis of HepG2 RNA isolated from cells exposed to cytokines. a) Autoradiograph showing a protected fragment (→) for the control (lane 1), IL-1β (lane 2), IL-6 (lane 3), and TNFα (lane 4) treated samples. b) The density of the signals of samples treated with IL-1β and TNFα was lower than for the control, suggesting down-regulation of Epo expression by these cytokines. There was no difference in the density of the control and IL-6-treated sample.

3.4 DISCUSSION

Interleukin-1ß, IL-6, and TNFα were found to inhibit Epo synthesis by HepG2 cells in culture (Chapter 2). This finding led to the investigation into the mechanism underlying this inhibition. Protein synthesis can be altered by extracellular modulators either during transcription of the gene into mRNA or it can be a post-translational event (Klausner and Harford, 1989; Darnell *et al.*, 1990). A common method of assessing whether transcription of a gene is affected is to determine its mRNA concentration. Northern blot analysis is the most frequently employed technique to quantify mRNA. The procedure typically involves electrophoresis of RNA through a 1% agarose/formaldehyde gel, followed by blotting onto nylon or nitrocellulose membranes which are then probed with a complementary DNA sequence that has been radioactively labelled. The blot is eventually autoradiographed after extensive washing to remove background radioactivity (Sambrook *et al.*, 1989).

Northern blots of cellular RNA extracted from HepG2 cells were first analyzed with a probe synthesised from highly conserved regions in exons IV and V of the published mouse sequence. As control, RNA isolated from the murine erythroleukemic cell line, IW32, was also included on the blots. The labelled probe was capable of hybridising to Epo transcripts in IW32 but not in HepG2 RNA samples (Figure 3.4a). This suggested that the probe could either not cross-hybridise with human Epo mRNA, or that the level of expression of the Epo gene in HepG2 cells was too low for its transcripts to be detected by Northern blotting.

When the human Epo cDNA was eventually obtained and used to probe Northern blots, it hybridised only to IW32 Epo transcripts (Figure 3.5). This proved conclusively that Epo mRNA in HepG2 cells was rare. The Epo mRNA was eventually detected in a sample

of enriched poly(A)$^+$ HepG2 RNA. Enriching numerous RNA samples is time-consuming and expensive. Large quantities (800 to 1000 µg) of total RNA is usually required to obtain a few micrograms (5 to 20 µg) of poly(A)$^+$ RNA. The amount of RNA that can be extracted from cell cultures is also extremely variable. As little as 30 µg is sometimes obtained from 10^7 cells (Sambrook *et al.*, 1989). Special precautions need to be taken when isolating poly(A)$^+$ RNA to prevent its degradation by ribonucleases. These inherent difficulties of Northern blot analysis prompted the search for an alternative technique.

Ribonuclease (RNase) protection assays are 20 times more sensitive than Northern blotting (Sambrook *et al.*,). They can detect as little as 0.1 pg of an mRNA species. Poly(A)$^+$ RNA is also not required. In this technique an RNA molecule, complementary to the target sequence, is used as probe and hybridisation is carried out in solution. Normal mRNA, also referred to as sense RNA, is transcribed from the 3' to 5' strand of a double stranded DNA molecule. The RNA probe, or riboprobe, used in protection assays is synthesised from the 5' to 3' strand of DNA. It is thus complementary to normal or sense RNA and can hybridise to it. RNA transcribed from the 5' to 3' DNA strand is also referred to as antisense RNA.

The antisense probe was transcribed from the *PstI/SacI* fragment of the rat Epo genomic DNA and labelled with α^{32}P-UTP (Amersham). The size of the probe was 132 bp which corresponded to the size of the exonic sequence of the DNA fragment. Ten-fold different amounts of IW32 cellular RNA were first probed with the antisense RNA. Autoradiography after three days exposure at -70°C showed a 132 bp protected fragment only for the 50 µg RNA sample (Figure 3.7). Lower amounts of RNA failed to yield a hybridisation signal. Samples of 50 µg cellular RNA were, therefore, used in the assays for Epo mRNA concentrations in control and cytokine-treated cultures of HepG2 cells. Figure 3.8a is a representative

autoradiograph of the results of such a protection analysis after 17 days exposure at -70°C. A protected fragment of approximately 132 bp in size was obtained for each sample. The density of the hybridisation signals obtained for the RNA samples extracted from cultures treated with IL-1ß and TNFα was lower than for the control signal. This suggests that these two cytokines suppress Epo synthesis by inhibiting transcription of the Epo gene. A similar finding was reported by Faquin *et al.*, (1992) who studied the effects of cytokines on hypoxia-induced Epo synthesis by Hep3B cells. There was no difference in the density of the control signal and the one generated by the RNA sample isolated from cells treated with IL-6. The decrease in the biosynthesis of Epo induced by IL-6 was, therefore, a post-transcriptional event.

In conclusion, these results support the idea that IL-1ß and TNFα can induce a blunted erythropoietin response by interfering with the transcriptional process of the Epo gene. Interleukin-6, a weak inhibitor of Epo production, appears to exert its activity either during translation or at a post-translational level.

3.5 COMMENTARY

The figures which appear in this chapter represent the best of eight to fifteen results. Numerous Northern blots were run in order to define appropriate stringency conditions, hybridisation buffers, hybridisation temperatures, probe concentrations, sample RNA concentrations, and exposure times. Probes were initially labelled using nick translation. The specific activity of these probes, however, proved to be too low and even failed to detect Epo mRNA of IW32 cell cultures. For protection assays, ^{35}S-labelled probes were also used but without success. The decay energy of ^{35}S was probably too low since an adequate signal could

not be obtained, even after exposing the autoradiographs for two months. This was originally done to contain costs since ^{35}S has a longer half-life than ^{32}P. Several RNase protection assays also had to be done in order to define optimum conditions.

CHAPTER FOUR

ERYTHROPOIESIS AND ERYTHROPOIETIN SYNTHESIS DURING ACUTE ASEPTIC INFLAMMATION

4.1 INTRODUCTION

The response of higher organisms to disturbances of their homeostasis due to infection, tissue injury, neoplastic growth, or immunologic disorders is called the acute phase response(APR) (Kushner, 1982). As schematically shown in Figure 4.1, it consists of an initial local reaction at the site of injury followed by a systemic reaction. The local reaction is characterized by a number of responses such as aggregation of platelets and clot formation, dilatation and leakage of blood vessels, and an accumulation and activation of granulocytes and mononuclear cells. After stimulation, these cells release inflammatory cytokines. These act on specific receptors on different target cells leading to a systemic reaction, which is characterized by fever, leucocytosis, increase in erythrocyte sedimentation rate, increases in secretion of adrenocorticotrophic hormone (ACTH) and glucocorticoids, activation of complement and clotting cascades, and decreases in serum levels of iron and zinc. Furthermore, drastic changes in the concentration of some plasma proteins occur. These plasma proteins, which are synthesized in the liver, are referred to as acute phase proteins or reactants (Castell *et al.*,1989; Kushner, 1982).

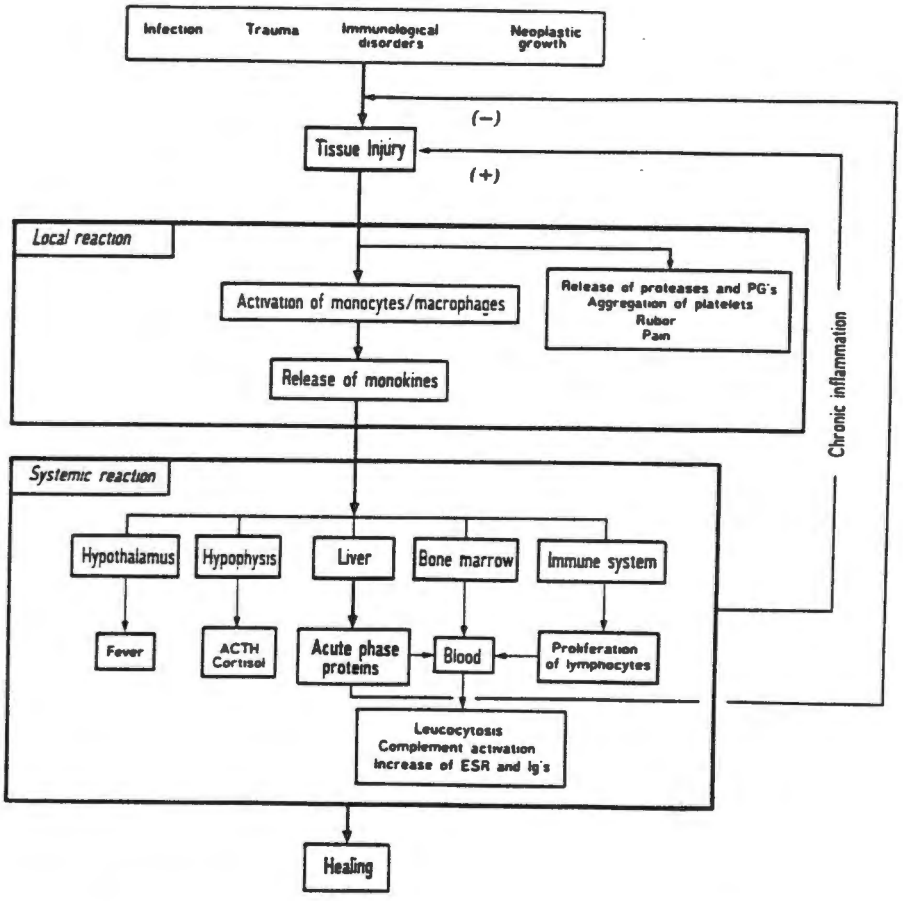


Figure 4.1. The acute phase response
(Adapted from Castell *et al.*,1989)

The acute phase reactants fulfil essential functions by neutralizing harmful consequences of injury (Pepys and Baltz, 1983). For example, proteinase inhibitors such as α_1-antitrypsin, α_1-antichymotrypsin, α_1-macroglobulin, α_2-macroglobulin, and α_1-inhibitor III antagonize the activity of proteinases released from macrophages and granulocytes (Gordon, 1976; Lonberg-Holm *et al.*, 1987; Pepys and Baltz, 1983). Fibrinogen participates in blood clotting and wound healing (Furie and Furie, 1988). C-reactive protein (CRP) and serum amyloid P (SAP) efficiently bind negatively charged molecules such as phosphorylcholine, a component of plasma membranes (Pepys and Baltz, 1983). Thus CRP opsonizes bacteria, parasites and plasma membranes, facilitating their clearance by phagocytic cells.

The whole spectrum of changes in gene expression of acute phase

proteins during inflammation is not caused by the action of a single factor. Among all cytokines secreted by monocytes and macrophages, those shown to be involved in the APR are IL-1, TNFα, and IL-6 (Ramadori *et al.*, 1985; Perlmutter *et al.*, 1986; Darlington *et al.*, 1986; Castell *et al.*, 1988; Morrone *et al.*, 1988). Together with these factors, glucocorticoids appear to act as important cofactors for the induction or repression of APR genes both *in vivo* and *in vitro* (Figure 2.2) (Baumann *et al.*, 1987).

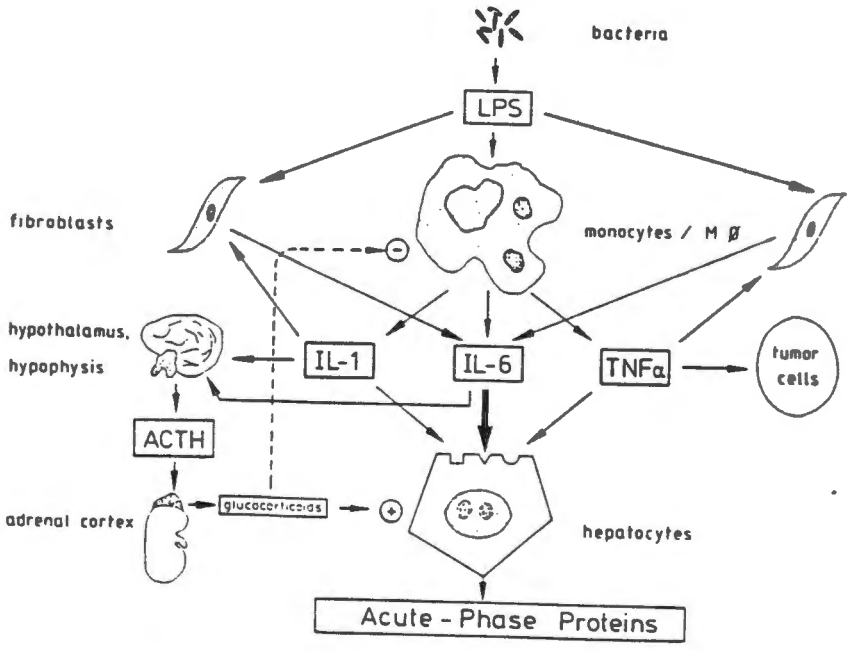


Figure 4.2. Regulation of hepatic acute phase protein synthesis by inflammatory mediators. (Adapted from Castell *et al.*, 1989)

Recombinant IL-1 induces some acute phase genes such as serum amyloid A (SAA), human C3 and factor B, and decreases the synthesis of albumin, but does not exert any effect on other acute phase reactants such as CRP or haptoglobin (Ramadori *et al.*, 1985; Perlmutter *et al.*, 1986; Darlington *et al.*, 1986). Tumour necrosis factor has an even more restricted spectrum of activity. This cytokine has been shown only to induce complement

C3 and factor B and to a certain extent α_1-antichymotrypsin (ACH)(Darlington *et al.*, 1986; Perlmutter *et al.*, 1986). Interleukin-6 has been identified as the major modulator of the APR (Castell *et al.*, 1989). Recombinant IL-6 is a potent inducer of the expression of fibrinogen, ACH, haptoglobin, factor B, haemopexin, SAA, CRP and α_1-acid glycoprotein (Baumann *et al.*, 1987; Gauldie *et al.*, 1987; Castell *et al.*, 1988; Morrone *et al.*, 1988).

Recombinant IL-1(Johnson *et al.*, 1989) and TNFα (Moldawer *et al.*, 1989; Tracey *et al.*, 1988) reportedly induce anaemia *in vivo*. Acute inflammation may, therefore, be accompanied by an anaemia induced by these two cytokines. Serum Epo levels would, thus, be expected to rise in order to resolve the anaemia. Since IL-1β, IL-6, and TNFα have been shown to inhibit *in vitro* Epo production (Chapter 2), the likelihood exists that these modulators of the acute phase response may also compromise the expected increased Epo synthesis *in vivo*. Erythropoietin could, therefore, behave like a negative acute phase reactant. Such a blunted Epo response would result in the persistence of the anaemia, as has been reported for chronic inflammation (Spivak *et al.*, 1989; Miller *et al.*, 1990).

In order to determine whether Epo expression is subject to modulation by mediators of the acute phase response, erythropoiesis and erythropoietin synthesis, were studied in an in *vivo* model for acute inflammation.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

Adult male Long-Evans rats weighing between 275 to 360 g were

used. Animals were kept in a well-ventilated room with a 12 h dark/light cycle and a thermostatically controlled temperature of 23 ± 1°C. The rats were group-housed (3 to 4) in polypropylene cages with metal rung tops and had free access to rat chow and water. One week prior to the experiment, animals were handled daily to reduce the effects of handling stress on the study. Sacrificing of animal groups took place in a separate room from the one in which the remainder of the animals were kept.

4.2.2 INDUCTION OF ACUTE INFLAMMATION

A well-established model for inducing acute inflammation, is by the subcutaneous injection of turpentine (Williams and Johnson, 1976; Schreiber *et al.*, 1982). Turpentine (Alchemist, SA) was sterilized with a 0.22 µm filter (Millipore, UK), and administered at a dose of 0.5 ml per 100 g body mass. Control animals received the same dose of sterile pyrogen-free isotonic saline (Sabax, SA).

At intervals of 0, 8, 12, 16, 24, 36, and 48 h after injection, groups of control and treated rats were anaesthetized with ether, their thorax surgically opened, and blood collected via cardiac puncture with a 26 gauge needle and 10 ml syringe. Between 5 and 8 ml blood was usually obtained from each animal. Approximately 2 ml was transferred to sterile 5 ml tubes containing a solution of the anticoagulant, EDTA. This was used for haematological determinations. The remainder of the blood was transferred to additive-free tubes for serum isolation. Groups of three to four animals were used per time interval.

4.2.3 HAEMATOLOGICAL MEASUREMENTS

Haemoglobin (Hb), haematocrit (Hct), red cell count (RCC), and white cell count (WCC) values were determined with a Coulter counter (Coulter Electronics, Model S-Plus). The mean cell volume (MCV), mean cell haemoglobin (MCH), and mean cell haemoglobin concentration (MCHC) were automatically calculated by the instrument from the Hb, Hct, and RCC values. Measurements were generally performed within 6 h of collecting the blood specimens.

4.2.4 PREPARATION OF SERUM

Whole blood was allowed to clot at room temperature for 1 h. The clot was disrupted with a blunt wooden applicator to release the serum and the sample centrifuged (Sigma, Model 3E-1) at 3000 rpm for 10 min. The serum was transferred to 1.5 ml Eppendorf tubes and stored at -40°C until required.

4.2.5 PROTEIN ESTIMATION

Total serum protein was assayed using the Bio-rad™ dye-binding microassay kit (Bio-rad, UK). Briefly, to 5 µl of serum was added 200 µl of dye reagent (diluted 1:4 with water) plus 795 µl of distilled water. Four 200 µl aliquots of each sample were transferred to a 96-well plate (Sterilin, UK) and their absorbances read at 640 nm on an ELISA plate reader (Cambridge Technologies, Model 7520). Bovine serum albumin (fraction V)[Sigma] was used as standard.

4.2.6 ALBUMIN DETERMINATION

Serum albumin levels were determined using a dye-binding method (Grant and Kachmar, 1976) with minor modifications. A bromocresol green working solution (Appendix) served as dye reagent. This reagent was diluted (1:6) with distilled water prior to use. To 25 µl of serum was added 5 ml of diluted dye reagent. This was mixed on a vortex, allowed to stand for 10 min at room temperature, and the absorbance read on a spectrophotometer (Beckman, Model DB) at 640 nm against the dye reagent as blank. Bovine serum albumin (Sigma) served as reference standard.

4.2.7 ERYTHROPOIETIN DETERMINATION

Serum Epo concentrations were determined using a commercially available radioimmunoassay (RIA) kit (Incstar, USA). The RIA procedure is a competitive binding disequilibrium radioimmunoassay which utilizes recombinant human Epo for both tracer and standards. Samples were incubated with the primary goat antibody and allowed to react for two hours before the tracer labelled with ^{125}I was added. Following an overnight incubation, the donkey anti-goat secondary antibody was incubated with standards or samples, primary antibody and tracer, for thirty minutes before the test tubes were centrifuged at 1600*g* to separate the bound from the unbound tracer. The unbound tracer was removed by decanting the supernatant from each test tube. The bound tracer in the remaining secondary antibody complex pellets was counted in a gamma counter (Packard, Model B5412). The ^{125}I counts are inversely proportional to the amount of Epo present in each sample. Although the primary antibody of the kit was raised against human Epo, studies done in our laboratory have shown cross-reactivity with rat Epo.

4.2.8 TUMOUR NECROSIS FACTOR-*alpha* DETERMINATION

Serum TNFα levels were measured with a commercially available ELISA kit (Genzyme, USA). The assay is a solid-phase enzyme immunoassay employing the multiple antibody sandwich principle. A hamster monoclonal antibody specific for murine TNF was coated on microtitre wells in a 96-well plate. Tumour necrosis factor present in standard, samples and unknown specimens was captured by the solid-phase monoclonal antibody. A goat polyclonal anti-murine TNF antibody, which binds to multiple epitopes on the TNF contained on solid phase, was added. A third antibody, horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin, was used to bind to anti-murine TNF:murine complexes. The peroxidase enzyme reacts with peroxidase substrate and OPD (chromagen) to produce colour which was proportional in intensity to the amount of TNF present.

Colour intensity was quantified by measuring absorbance at 492 nm using an ELISA reader (Multiskan, MCC 340). A reference curve was obtained by plotting the TNF concentration of several dilutions of standard versus absorbance. The TNF concentrations in experimental samples were read off the reference curve. According to the manufacturer the antibodies in the test, in spite of being raised against mouse TNFα, can detect rat TNF as well.

4.2.9 HISTOLOGY

Within 12 to 24 h after turpentine treatment, rats developed a solid lump at the site of injection. The lump was surgically removed from three such animals at the time of sacrifice, and transferred to containers with fixative (Appendix).

The tissues were dehydrated and processed as follows:

i) 2 x 6 h incubations at room temperature in 70% ethanol. This step was repeated.

ii) 1 x 16 h incubation in 90% alcohol.

iii) 3 x 2 h incubations in absolute ethanol.

The dehydrated tissues were embedded in wax by placing them in xylene overnight and transferring them to paraffin wax at 60°C for 1 h. The latter step was repeated thrice. Finally, the tissues were transferred to fresh wax which was allowed to solidify at 4°C overnight. Tissues blocks were sectioned with a microtome (Jung AG, Model 29988) and the sections (4 µm in width) mounted on a glass slide. The sections were dewaxed by placing the slides in xylol for 10 min and then hydrated as follows:

i) 3 x 1 min changes in absolute alcohol,

ii) 2 x 1 min changes in 90% alcohol, followed by

iii) 1 x 1 min change in 70% alcohol and 1 x 1 min change in 50% alcohol.

iv) The sections were rinsed in running tap water for 1 to 2 min.

Each section was stained with haematoxylin and eosin by immersing the slides in haematoxylin for 10 min, washing in running tap water for 2 to 3 min, followed by differentiation in 1% hydrochloric acid in 70% alcohol for a few seconds. This was stopped by washing in running tap water for 5 min. The slides were then transferred into 1% aqueous eosin for 10 min. Surplus stain was washed off with water and the sections dehydrated in alcohol and cleared in xylene as above. Finally, they were mounted in entellen mountant and examined microscopically.

4.2.10 STATISTICS

Data are represented as means plus standard error of the means (SEM). Statistical comparisons between group means at various time points within control and treated populations, as well as between control and treated means at corresponding time intervals, were performed using a two-way analysis of variance (ANOVA)(Bartlett's test). A *p* value less or equal to 0.05 was considered significant.

4.3 RESULTS

4.3.1 HISTOLOGICAL EXAMINATION

Figure 4.3 is a typical photograph of a cross section through the node which developed in rats after subcutaneous injection with turpentine. The lesion is filled with leucocytes, typical of a turpentine-induced granuloma (Rocha e Silva and Leme, 1972).

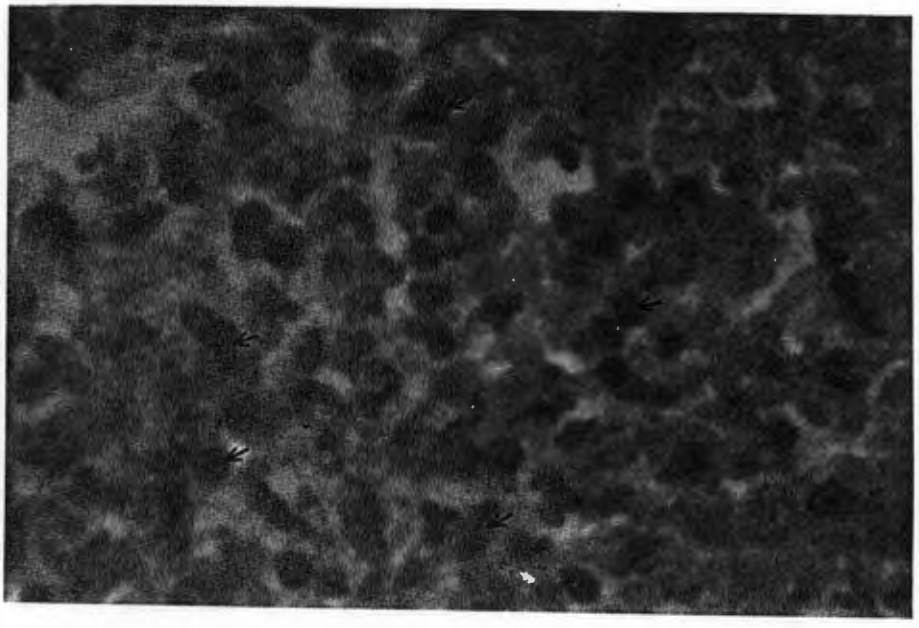


Figure 4.3. Granulomatous lesion which developed at the site of turpentine injection. Note the numerous leucocytes (→).

4.3.2 SERUM PROTEIN CONCENTRATIONS

Protein concentrations in groups of control and treated rats are depicted in Figure 4.4. The average protein level remained unchanged in both control and treated animals throughout the duration of the experiment. There was also no statistical difference between corresponding groups of treated and untreated rats.

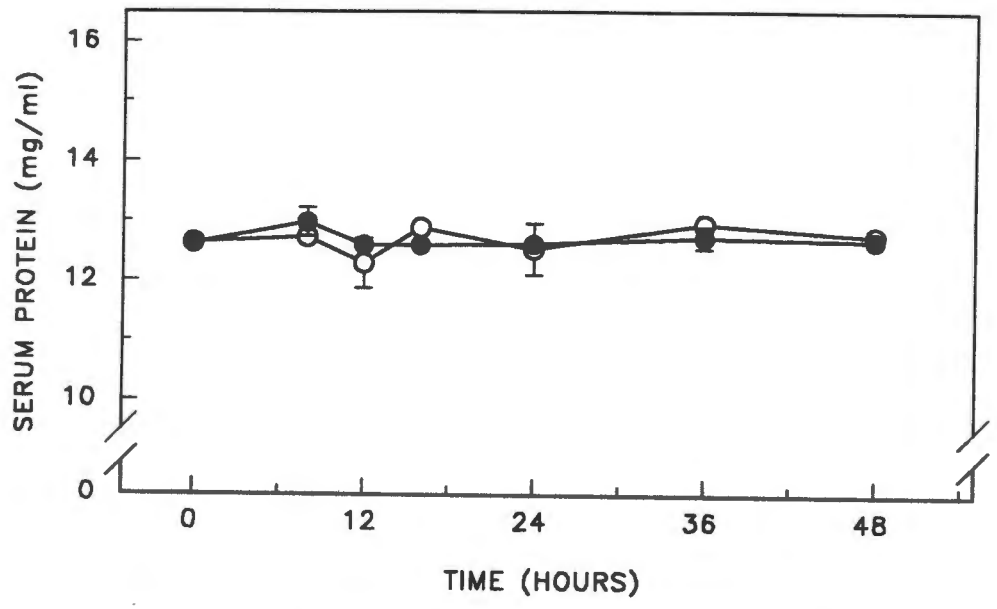


Figure 4.4. Total serum protein concentrations in control(○) and treated (●) rats at different time intervals after treatment.

4.3.3 SERUM ALBUMIN

The mean serum albumin concentrations in the treated animals were relatively constant over the first 24 h (Figure 4.5). It started to decline, thereafter, with the levels in the 36 and 48 h groups being significantly less than in the 0 h group ($p \leq 0.05$). No fluctuations in serum albumin levels were detected in control groups. The concentration of serum albumin in the 36 and 48 h treated groups were also significantly less then in the corresponding control groups ($p \leq 0.05$).

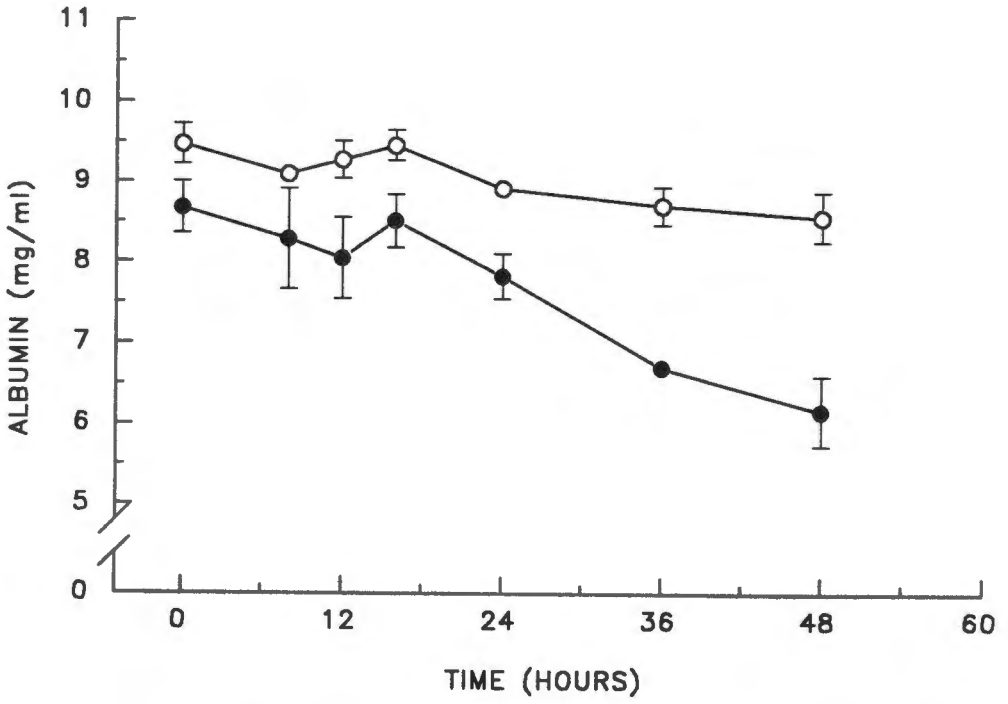


Figure 4.5. Serum albumin concentrations in control(o) and treated (●) rats at different time intervals. (The SEM of certain data points are smaller than the symbols)

4.3.4 HAEMATOLOGICAL PARAMETERS

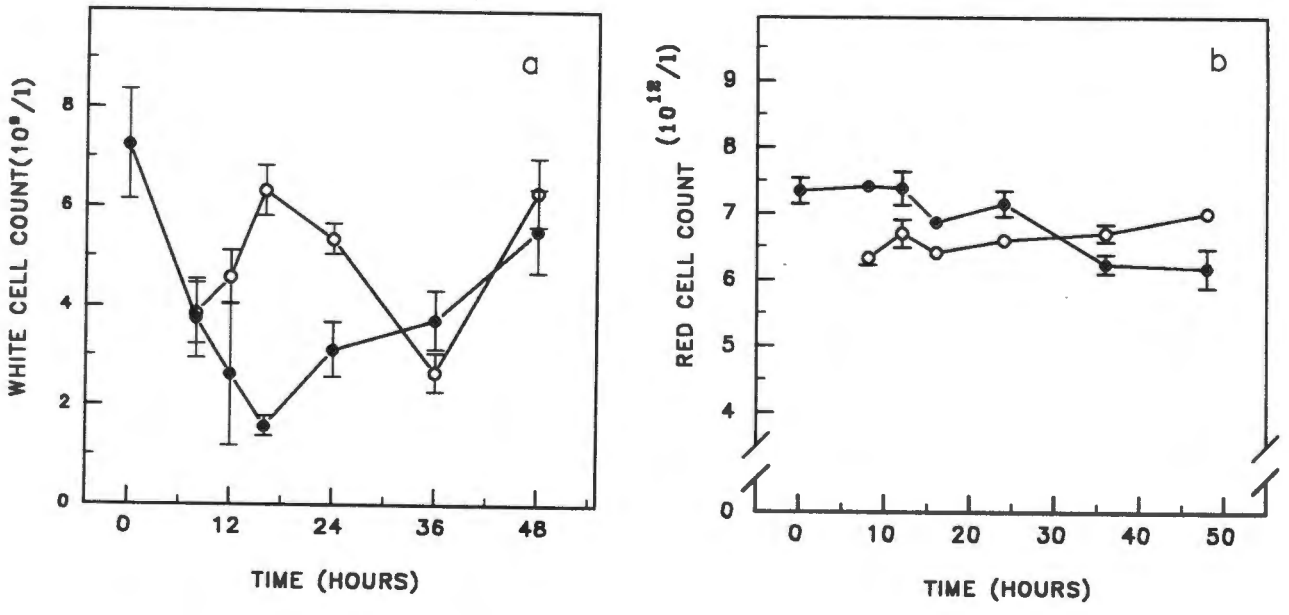
Total leucocyte counts dropped sharply over the first 16 h following turpentine injection (Figure 4.6a). The counts differed significantly from those of corresponding control groups($p \leq 0.05$). Thereafter, the WCCs gradually increased until they reached normal levels by 36 h.

The red cell counts (RCCs) and haematocrits (Hcts) remained relatively constant over the first 24 h in both treated and untreated groups (Figures 4.6b and 4.6c). Both parameters declined in the turpentine-treated group thereafter, with the values at 36 and 48 h differing significantly from the group at 0 h ($p \leq 0.05$). The mean RCCs and Hcts of the treated groups at 36 and 48 h were, however, not significantly different from those

of the corresponding control groups.

The average Hb values, as was the case with the RCCs and Hcts, did not undergo significant changes over the first 24 h (Figure 4.6d). A rapid decrease occurred in treated animals thereafter, so that the Hb values of the groups at 36 and 48 h were significantly different ($p \leq 0.01$) compared to the one at 0 h. Unlike the case with the RCCs and Hcts, the Hb values of the 36 and 48 h treated groups were also significantly less ($p \leq 0.05$) than those of corresponding control groups.

The MCV, MCH, and MCHC values did not undergo any significant changes in treated or untreated groups over the time course of the experiment (Figures 4.6e, 4.6f, 4.6g). These parameters also did not differ between treated and untreated animals.



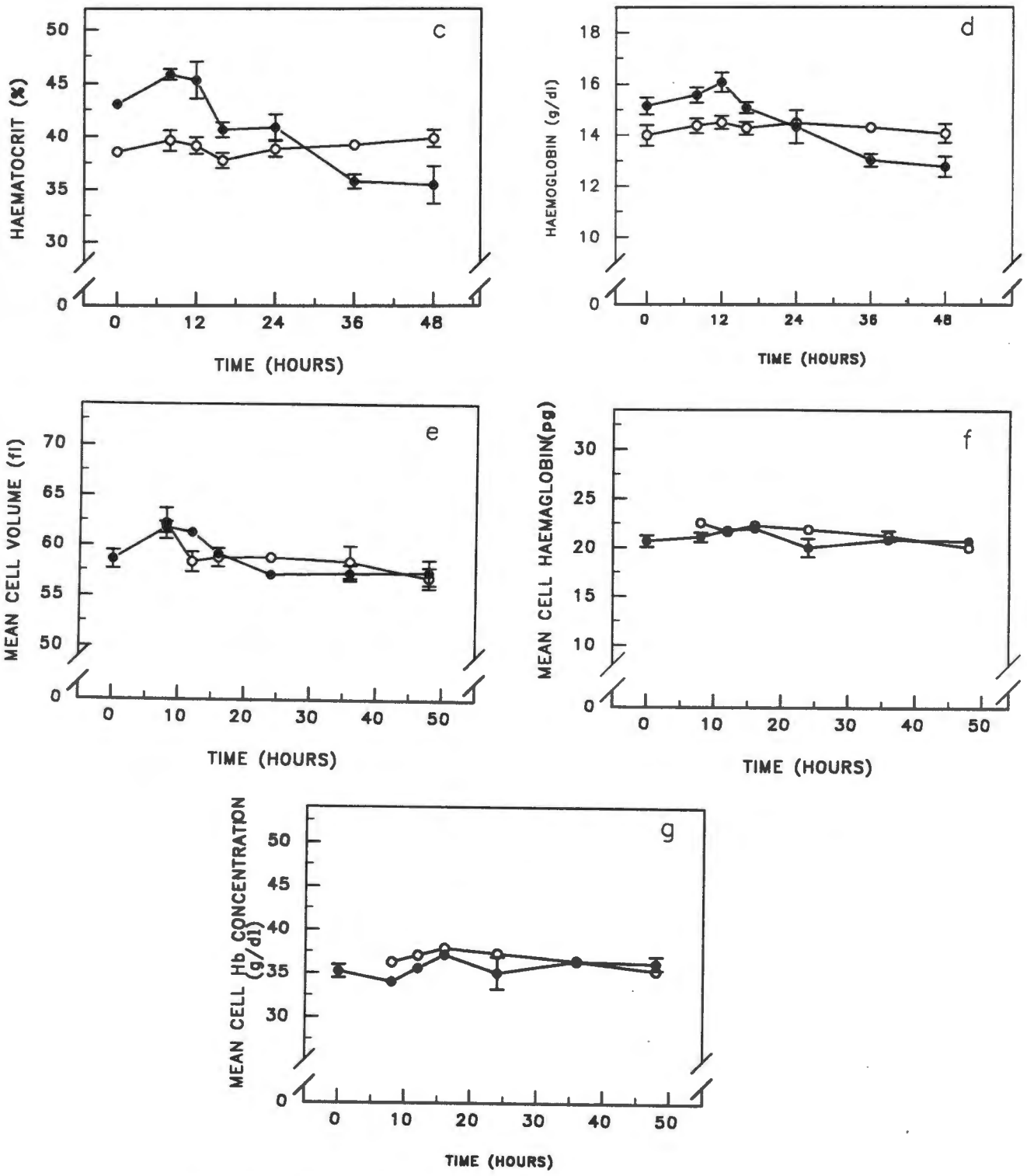


Figure 4.6. Haematological status of control(o) and treated (●) groups. a) White cell count (WCC); b) Red cell count (RCC); c) Haematocrit (Hct); d) Haemoglobin (Hb); e) Mean cell volume (MCV); f) Mean cell haemoglobin (MCH) and g) Mean cell haemoglobin concentration (MCHC). SEM of certain data points are smaller than the circles.

4.3.5 ERYTHROPOIETIN

Fluctuation in the serum levels of Epo was observed in both treated and control rats over the first 24 h of the experiment (Figure 4.7). No statistical difference in the concentration of the hormone between control and treated groups was observed during this period. Thereafter, the level of serum Epo in the treated animals started to increase so that its concentration in the groups at 36 and 48 h was significantly higher than in corresponding control groups ($p \leq 0.05$).

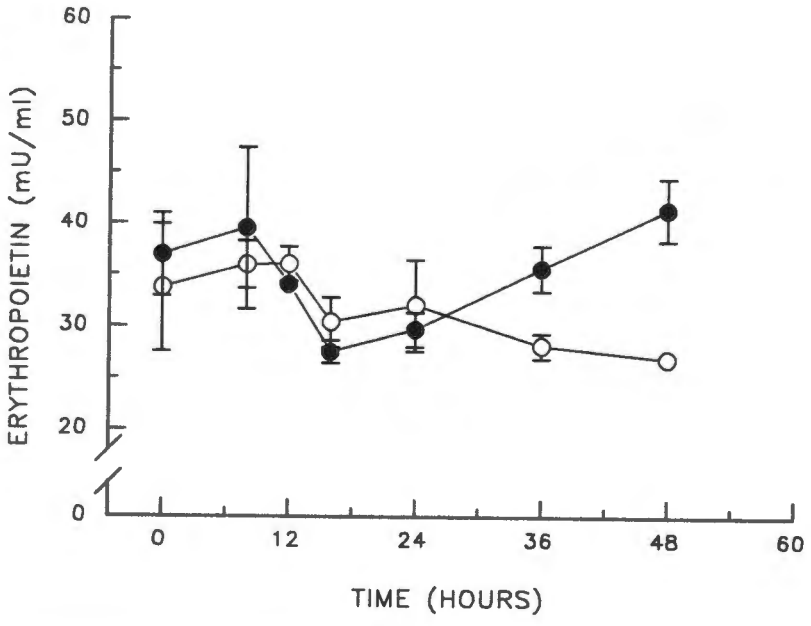


Figure 4.7. Serum erythropoietin levels in control(o) and treated (●) rats following treatment with saline and turpentine, respectively.

4.3.6 TUMOUR NECROSIS FACTOR-*alpha*

Serum TNFα levels started to rise 24 h after turpentine administration and remained elevated until the experiment was terminated (Figure 4.8). The concentrations of this cytokine in

the 36 and 48 h groups differed significantly from that of the group at 0 h ($p \leq 0.05$).

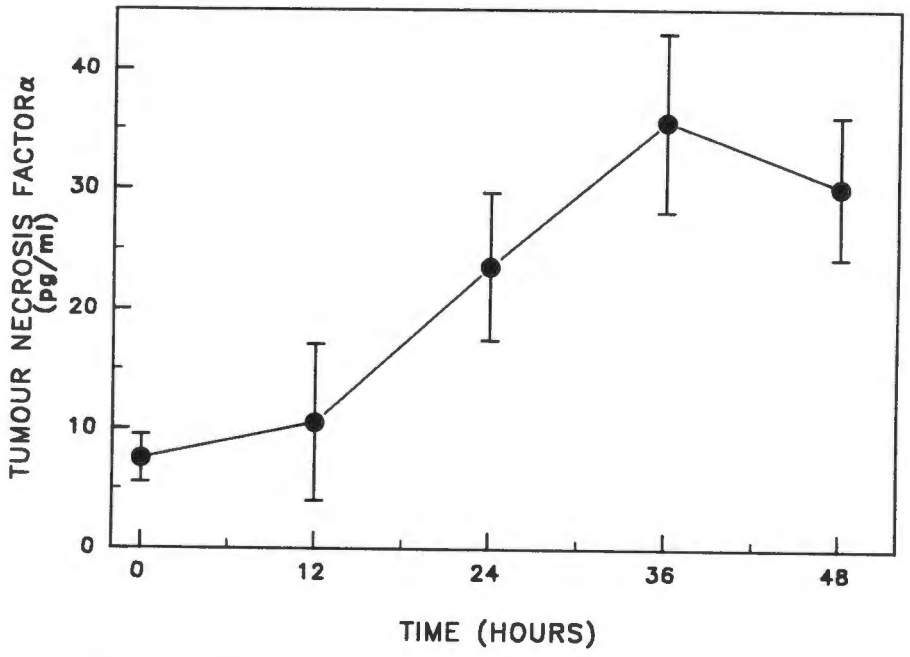


Figure 4.8. Serum TNFα levels in treated groups at 12 h intervals following turpentine administration.

4.4 DISCUSSION

In this investigation, the effect of acute inflammation on erythropoiesis, in general, and on erythropoietin synthesis, in particular, was assessed. The well-established turpentine model of acute inflammation was used (Williams and Johnson, 1976; Schreiber *et al.*, 1982).

Serum albumin concentrations were found to decrease significantly ($p \leq 0.05$) 36 h after turpentine treatment and continued to decline, thereafter (Figure 4.5). In contrast, the level of this protein in the serum of control animals remained relatively unchanged. Albumin is a negative acute phase reactant whose concentrations in serum, along with its synthesis in the liver, usually decrease during acute inflammation

(Northemann *et al.*, 1983; Dowton and Colten, 1988). The reason for the delay in the drop in serum albumin concentration was due to the indirect action of turpentine. A cascade of reactions, including activation of monocytes and macrophages, the formation, secretion, and transport of inflammatory mediators from the local abscess to the liver, has to proceed before changes in acute phase protein responses can occur. This is borne out by the observation that significant TNFα levels appeared in the serum only 36 h after turpentine administration (Figure 4.8). Acute phase protein responses usually occur much earlier when animals are treated with the inflammatory mediators directly (Geiger *et al.*, 1988). The observed reduction in serum albumin levels subsequent to turpentine treatment suggests, therefore, that an acute phase response was elicited in treated animals.

Total serum protein levels in treated animals did not differ significantly from those in controls (Figure 4.4). This was expected since the decrease in serum concentrations of negative acute phase proteins is compensated by an increase in the levels of positive acute phase reactants. The overall change in total serum protein levels during acute inflammation, therefore, remains negligible (Schreiber *et al.*,1989).

An initial drop in WCC was observed in treated animals (Figure 4.6a). This may be due to the migration of white cells from the systemic circulation to the site of injection. Histological examination of the local abscess which developed in response to the turpentine, showed the presence of a large number of small white blood cells. A gradual increase in this parameter started to occur in the 24 h treated group and by the end of the experiment the WCC value was the same as that in controls. This probably represents enhanced proliferation of white blood cells in the bone marrow followed by an increased influx into the blood. As was the case with serum albumin, the RCC, Hct, and Hb

values showed significant decreases only 36 h after turpentine injection. This was about the same time that significant TNFα levels were detected in the serum. Tumour necrosis factor-*alpha* and IL-1 have been shown to antagonize bone marrow erythropoiesis (Moldawer *et al.*, 1989; Schooley *et al.*, 1987). The reduction in the values of these haematological parameters could, therefore, be a reflection of the inhibitory action of these cytokines on erythropoiesis during the inflammatory process.

The calculated haematological parameters, MCV, MCH, and MCHC, did not undergo any changes in response to turpentine-induced acute inflammation. Alterations in these parameters are usually observed during anaemia when they are used to classify or describe the condition in terms of the size of red blood cells, as well as the haemoglobin content of the erythrocyte (Dacie and Lewis, 1975). Since no changes were observed in these parameters in the treated group and, since the decreases in the RCC and Hct values were not significantly different from corresponding control animals, the reduced erythropoiesis which developed in response to the acute inflammation cannot be classified as anaemia. Nevertheless, the reduction in the number of circulating red blood cells and, particularly the drop in haemoglobin in the 36 and 48 h treated groups, were sufficient to effect an increase in the level of serum Epo in these animals (Figure 4.7). Prior to the increase, Epo levels in treated animals fluctuated in the same manner as in control rats, being high in one time-group and then low in the next one. Such a pattern is suggestive of a rhythmic synthesis of the hormone. In fact, it has been reported that the production of Epo is subject to a circadian or diurnal rhythm in man (Miller *et al.*, 1981; Wide *et al.*, 1989). It is, therefore, likely that such a mode of biosynthesis may also occur in rats. The cyclical mode of production was, however, abolished in those rats with lowered red cell indices in which a need existed for

enhanced Epo synthesis to correct the haematological status of the animals.

The findings obtained in this study suggest that erythropoiesis is antagonized during acute inflammation. Such antagonism is most likely mediated by IL-1 and TNFα. Both these cytokines have been shown to antagonize red blood cell production *in vivo* (Schooley *et al.*, 1987; Moldawer *et al.*, 1989). It was also found that those animals with lowered RCC, Hct, and Hb values responded by increasing their levels of serum Epo. It is not possible to conclude from the data, however, whether Epo synthesis was affected by any of the mediators of the acute phase response. All three acute phase mediators (IL-1β, TNFα, and IL-6) were shown to inhibit Epo production *in vitro* (Chapter 2). The likelihood, therefore, exists that the increased Epo levels detected in the serum of rats with a reduced erythropoiesis may not be optimal. It was for this reason that the study was repeated, but with rats which were stressed haematologically to ensure optimal production of the hormone. If the inflammatory mediators could inhibit Epo synthesis *in vivo*, then the optimal Epo response in treated animals should be less than in anaemic controls.

4.5 THE EFFECT OF ACUTE INFLAMMATION ON OPTIMAL ERYTHROPOIETIN PRODUCTION IN HAEMATOLOGICALLY STRESSED RATS

4.5.1 INTRODUCTION

In the previous study, the erythropoietic response to aseptic acute inflammation in Long-Evans rats was examined. A slight, but statistically significant, decrease in certain haemopoietic indices (haemoglobin, haematocrit and red cell count) was noted which was accompanied by an enhanced serum erythropoietin concentration. Unfortunately, it could not be deduced whether the Epo response was optimal. It was demonstrated in Chapter 2 that the mediators of the acute phase response, notably IL-1β, IL-6, and TNFα can inhibit Epo biosynthesis *in vitro*. The likelihood exists, therefore, that these cytokines may also antagonize Epo synthesis during the APR. In order to ascertain whether Epo production was appropriate, experimental anaemia was induced in rats followed by the induction of acute inflammation. The Epo response in these animals was compared to a control group with a similar degree of anaemia, but without acute inflammation.

4.5.2 MATERIALS AND METHODS

4.5.2.1 INDUCTION OF ANAEMIA

Adult male Long-Evans rats weighing between 200 and 270 g were used. Haemolytic anaemia was induced by an intraperitoneal (i.p.) injection of phenylhydrazine hydrochloride (PZN) [Sigma],

at a dose of 60 mg/kg, on two consecutive days (Rencricca *et al.*, 1970; Hara and Ogawa, 1976).

4.5.2.2 INDUCTION OF ACUTE INFLAMMATION

Two days after PZN injection, rats were divided into two batches. One batch was injected with sterile turpentine (0.5 ml/100 g). The other batch received the same dose of sterile isotonic saline and served as control. At intervals of 12 h thereafter, groups of 3 to 4 animals from each batch were sacrificed and blood collected via cardiac puncture as before.

4.4.2.3 HAEMATOLOGICAL INVESTIGATION

The red cell indices, Hct, RCC, Hb, MCV, MCH, and MCHC were determined as before (section 4.2.3). Erythropoietin concentrations were measured in serum with the RIA kit described in section 4.2.7.

4.4.2.4 STATISTICAL ANALYSIS

Data are represented and were analysed in the same manner as in the previous study. A p value less or equal to 0.05 was considered significant.

4.5.3 RESULTS

4.5.3.1 HAEMATOLOGICAL PARAMETERS

The Hb (Figure 4.9a) and Hct (Figure 4.9b) of control and

treated groups sacrificed immediately after saline or turpentine injection, were significantly lower than the normal values (Hb: 14.1 ± 0.3 g/dl; Hct: 38.68 ± 1.632%) for Long-Evans (LE) rats ($p \leq 0.01$ for Hb of control rats; $p \leq 0.05$ for Hb of treated rats and Hct of both). These indices remained low in rats of both groups sacrificed at 12 and 24 h after injection. Thereafter, control groups had Hb and Hct values which were significantly elevated above those of earlier groups ($p \leq 0.01$). However, treated rats sacrificed at 36 and 48 h after turpentine administration had values which remained depressed and which were significantly less than corresponding controls ($p \leq 0.05$). Only in the 72 h group did treated rats show an increase in their Hb and Hct, although these did not differ significantly from the values obtained for earlier groups.

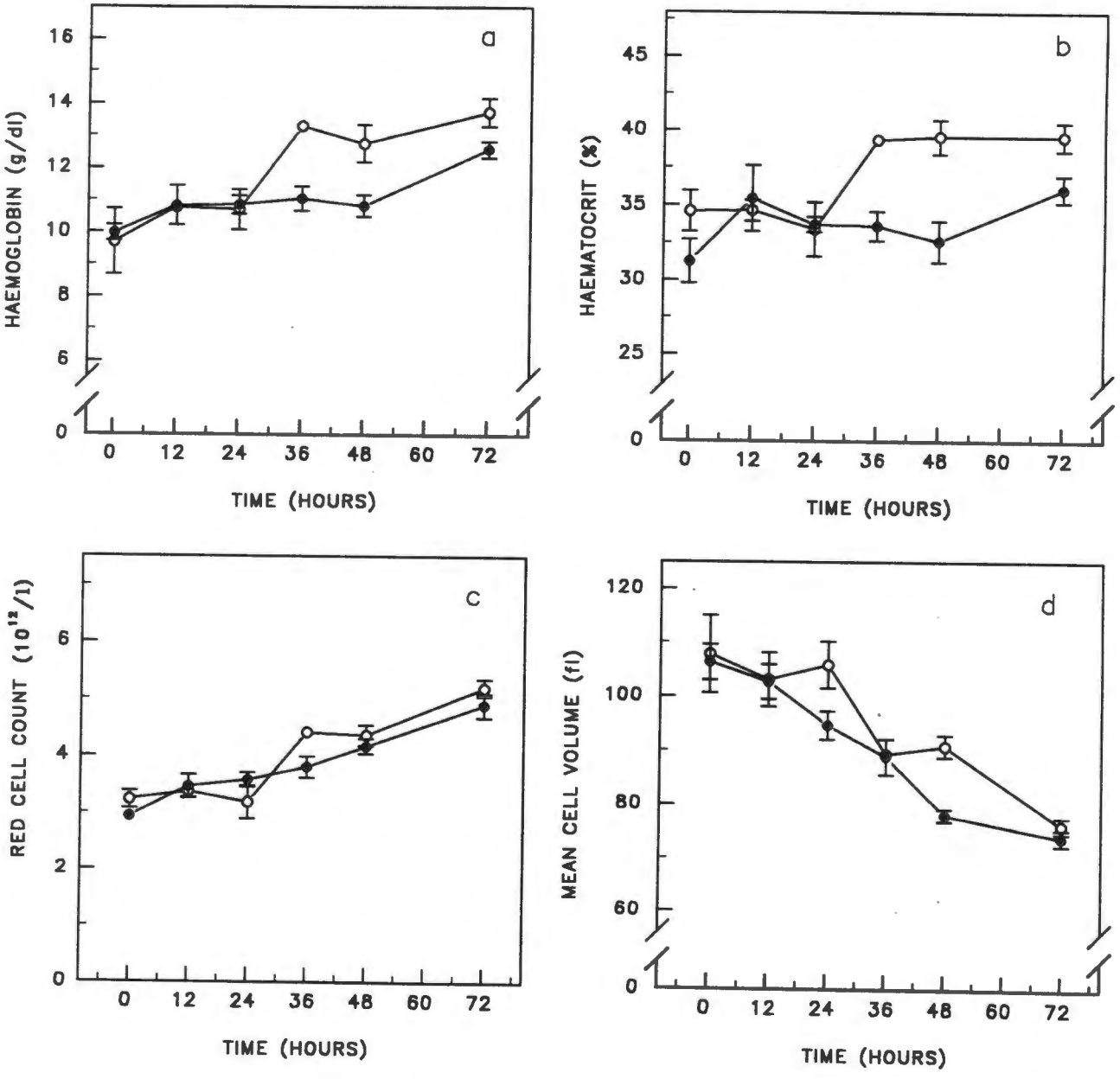
The RCCs in both control and treated animals were also initially lower than the normal RCC ($6.158 \times 10^{12} \pm 0.308 \times 10^{12}$/l) of LE rats ($p \leq 0.01$). However, the RCCs were higher in the 36 h control and treated groups, and continued to increase in groups sacrificed thereafter (Figure 4.9c). The differences in the RCC between treated and corresponding control groups were not significant.

The MCVs (Figure 4.9d) in both control and treated animals were initially higher than the normal value (62.76 ± 1.544 fl) for LE rats ($p \leq 0.01$). It started to decrease in subsequent groups, and continued to do so, until the experiment was terminated. Control and corresponding treated groups did not differ in their average MCV values.

The MCHs (Figure 4.9e) showed the same trend as the MCVs, being high in the first control and treated groups and decreasing in groups sacrificed thereafter. The MCHs in both control and treated animals were also substantially elevated ($p \leq 0.05$) above the normal value (22.88 ± 0.63 pg). Differences in the MCH

between control and treated groups at specific time intervals were not statistically significant.

The MCHCs (Figure 4.9f) in control and treated groups sacrificed immediately after saline and turpentine administration (0 h groups) were significantly lower than the normal value (36.44 ± 0.971 g/dl) for five LE rats ($p \leq 0.05$). The index gradually increased in both control and treated groups sacrificed at subsequent intervals. Values for MCHC in control groups did not vary significantly from those for corresponding treated groups.



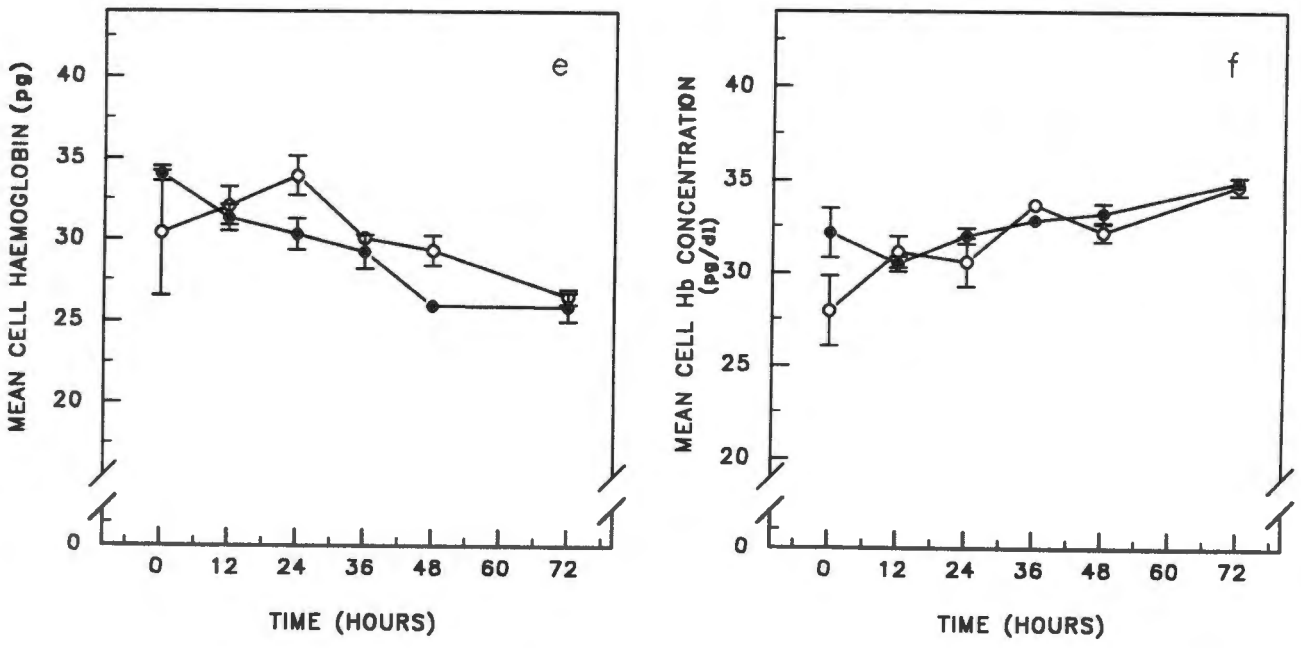


Figure 4.9. Haematological status of groups of control(o) and treated (●) anaemic rats at different time intervals following the administration of saline or turpentine, respectively. a) Hb; b) Hct; c) RCC; d) MCV; e) MCH and f) MCHC.

4.5.3.2 ERYTHROPOIETIN

Initial Epo levels (Figure 4.10) in control and treated rats were significantly elevated ($p \leq 0.01$) above the normal value (33.6 ± 5.8 mU/ml) measured in a group of five LE rats of similar mass and housed under identical conditions. The concentration of Epo remained high in control and treated animals sacrificed 12 and 24 h after treatment, with no difference between the corresponding groups. The level was lower in the 36 h control group and remained on a plateau in subsequent groups. The Epo concentrations measured in these animals were within the normal range and differed significantly ($p \leq 0.01$) from those sacrificed at earlier time-points.

In spite of the downward trend in the concentration of Epo in the 36 to 72 h treated groups, the levels did not differ statistically from those measured in groups at earlier intervals. The mean Epo concentrations of the 36, 48 and 72 h control groups were, however, significantly lower than those of corresponding treated groups ($p \leq 0.01$).

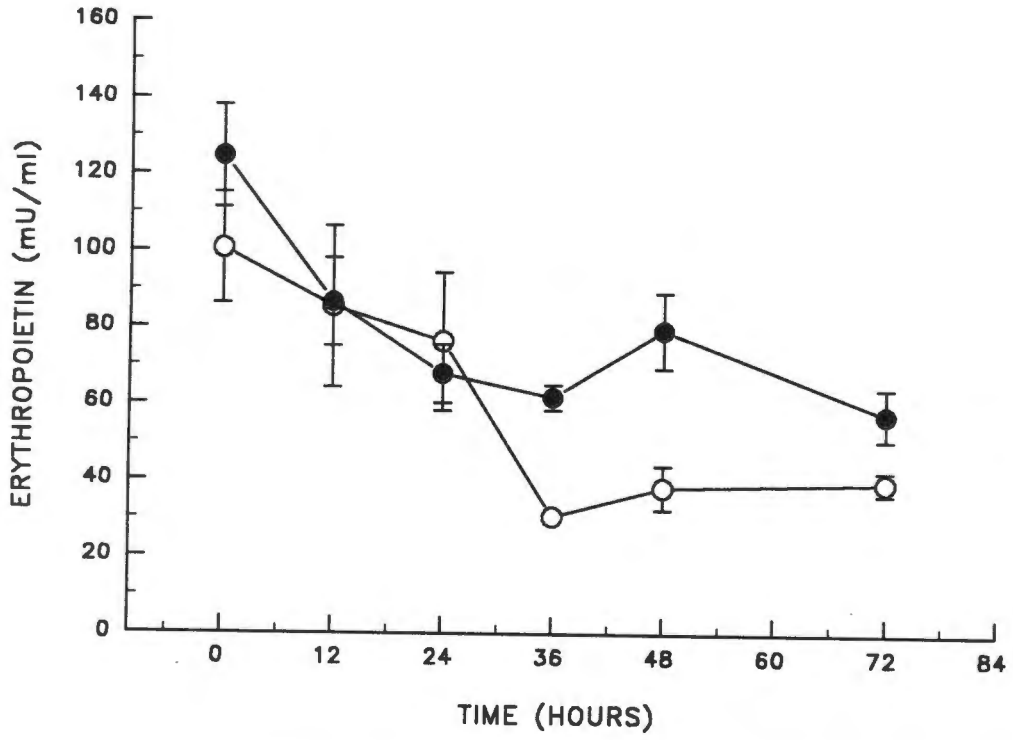


Figure 4.10. Serum Epo concentrations of anaemic control(○) and treated (●) rats at different time intervals following the injection of saline or turpentine, respectively.

4.5.4 DISCUSSION

The objective of this study was to determine whether the enhanced erythropoietin synthesis, observed during acute inflammation, was appropriate for the associated reduction in erythropoiesis. Mediators of the acute inflammatory response, notably IL-1β, IL-6, and TNFα, have been shown to inhibit Epo

production by the hepatoma cell line, HepG2 (Chapter 2). The *in vivo* synthesis of this hormone may, therefore, also be compromised during acute inflammation. This was investigated by comparing the Epo response, at intervals after inducing anaemia, in rats with and without acute inflammation.

Phenylhydrazine (PZN) produced a severe anaemia as evidenced by the low initial Hct, Hb, and RCC values in both control and treated rats. There was no difference in the values for these indices in control and treated rats sacrificed immediately after saline or turpentine administration (0 h interval). The initial degree of anaemia was, therefore, similar in both groups. The high initial MCVs and low MCHs in control and treated rats, describe a macrocytic anaemia which is characteristic of excessive erythropoiesis due to haemolysis (Jacobs and Bird, 1983; Dacie and Lewis, 1975).

The anaemia was accompanied by high levels of serum Epo in both control and treated animals. Groups, sacrificed at intervals over the first 24 h, had very high serum concentrations of the hormone with no differences in the levels between control and corresponding treated animals (Figure 4.10). Groups of control rats sacrificed subsequent to the 24 h interval had elevated RCC, Hct, and Hb values, indicating recovery from the anaemia. This occurred in response to the high concentrations of Epo which stimulated erythropoiesis. Resolution of the anaemia in turn lowered Epo production, causing serum levels of the hormone to decline in these groups.

In the case of treated animals, however, rats sacrificed after the 24 h interval continued to have low values for the three haematopoietic indices (Hb, Hct, and RCC) indicating the persistence of the anaemia. As expected, serum Epo levels remained elevated in these groups. The continued anaemia was most likely due to the suppression of erythropoiesis by the

cytokines, IL-1 and TNFα, elaborated during the APR. Interleukin-1 can antagonize the action of Epo on erythroid precursors (Schooley *et al.*, 1987), and TNFα has been found to inhibit erythropoiesis *in vivo* (Johnson *et al.*, 1989; Moldawer *et al.*, 1989; Broxmeyer *et al.*, 1986; Tracey *et al.*, 1988).

Apart from suppressing the proliferation and differentiation of erythroid precursors, the two cytokines, together with IL-6, have also been shown to inhibit the biosynthesis of Epo, albeit *in vitro*. It is not possible, however, to determine from the data whether the cytokines exerted any effect on the synthesis of the hormone. The reason being that inflammatory mediators initiated their effects 36 h after turpentine treatment, by which time, Epo synthesis had already passed its peak. As explained before, turpentine acts indirectly. Its administration is followed by a cascade of reactions, leading ultimately to the formation, secretion, and transport of acute phase cytokines into the circulation. This accounts for the lag period of approximately 36 h before their effects manifest. The detection of significant TNFα levels 36 h after turpentine injection (Figure 4.8) adds support to this reasoning.

Phenylhydrazine, on the other hand, appears to act rapidly in inducing anaemia. Maximal Epo synthesis must have occurred during the two day period prior to administering turpentine to treated rats. By the time the inflammatory cytokines started to exert their effects, active Epo synthesis was either terminated or down-regulated. Erythropoietin levels in control rats at this stage (36 h interval) were already within the normal range. The higher Epo levels in the treated groups sacrificed after the 24 h interval were most probably due to stimulated Epo synthesis in response to the cytokine-mediated reduction in erythropoiesis. Whether the Epo output was appropriate for the degree of anaemia, cannot be deduced, since Epo levels for corresponding control groups with the same degree of anaemia,

are lacking. It is interesting to note, however, that anaemia due to a chemical insult, unrelated to acute inflammation, can be prolonged by mediators of the acute phase response.

The two day treatment-free period, following PZN administration, was to allow the haemoglobin released from lysed erythrocytes to be excreted. The Coulter counter measures total haemoglobin concentrations only. By ensuring the excretion of free haemoglobin molecules, accurate Hb values for each animal could be obtained. This precaution, unfortunately, precluded any observation of possible cytokine effects on maximal Epo synthesis. The experiment was, therefore, repeated with the influx of cytokines into the systemic circulation of treated animals being synchronized with active Epo production.

4.6 THE EFFECT OF ACUTE PHASE CYTOKINES ON ACTIVE ERYTHROPOIETIN SYNTHESIS IN HAEMATOLOGICALLY STRESSED RATS

4.6.1 INTRODUCTION

The objective of this investigation was to determine whether the mediators of the acute phase response (APR) could antagonize the synthesis of Epo in anaemic rats. The experiments carried out thus far have revealed that aseptic acute inflammation induces a lowering in the values of the three basic haemopoietic indices (Hct, RCC, and Hb), with a concomitant increase in serum Epo concentrations. It was also found that acute inflammation can prolong existing anaemia in haematologically stressed animals. However, it has not been possible to conclude from the data whether optimum Epo synthesis in response to phenylhydrazine-induced anaemia, was affected by mediators of the APR.

The following experiment was designed to ensure that inflammatory mediators are present in the circulation of rats when active synthesis of Epo, in response to experimentally-induced anaemia, is occurring. This was achieved by inducing anaemia 36 h after turpentine administration. The effect of cytokines, which would appear in the circulation after the 36 h lag period, on maximal Epo synthesis could then be assessed and compared with Epo levels in rats having the same degree of haemolytic anaemia, but without acute inflammation. Phenylhydrazine effects anaemia within 12 to 24 h of administration (Rencricca *et al.*, 1970; Hara and Ogawa, 1976).

4.6.2 MATERIALS AND METHODS

4.6.2.1 INDUCTION OF ACUTE INFLAMMATION

Adult male Long-Evans rats, weighing between 225 and 375 g, were used. The animals were treated and housed under the same conditions as described in section 4.2.3. The rats were divided into two batches. One batch was injected subcutaneously with sterile turpentine (0.5 ml/100 g), and the other, which served as control, with the same dose of sterile apyrogenic isotonic saline. Blood was withdrawn, via cardiac puncture, from groups of three to four animals from each batch, at 12 h intervals. The animals were kept under ether anaesthesia during the procedure.

4.6.2.2 INDUCTION OF ANAEMIA

Thirty six hours after the initial treatment, all rats received a single i.p. injection of sterile PZN in isotonic saline (60 mg/kg). Groups of control and turpentine-treated rats continued to be sacrificed and their blood collected at intervals of 12 h until 72 h after the initial injection. The last group from each batch was sacrificed 24 h after the previous group, i.e., 96 h after the start of the experiment.

4.6.2.3 HAEMATOLOGICAL DETERMINATIONS

Haematocrits and RCCs were determined with a Coulter counter, as before. Accurate Hb values could not be obtained, since soluble haemoglobin from lysed erythrocytes was present in all blood specimens. The MCH and MCHC, which are calculated from the Hb value were, therefore, also inaccurate. The MCV was

automatically generated by the instrument. Erythropoietin concentrations were assayed in serum with the commercial RIA kit, as described in section 4.2.7.

4.6.2.4 STATISTICAL ANALYSIS

Data are represented as means ± SEM. Statistical comparisons were performed as described in section 4.2.10. As before, a p value less or equal to 0.05 was considered significant.

4.6.3 RESULTS

4.6.3.1 HAEMATOLOGICAL INDICES

The values of the Hct were within the normal range (38·7 ± 1·6%) for both control and treated groups over the first 36 h following saline or turpentine administration (Figure 4.11a). Thereafter, they declined and were significantly lower ($p \leq 0.05$) in control and treated groups sacrificed at the 48 h interval. The mean Hcts for groups of treated rats, sacrificed from the 48 h time interval until the end of the experiment at 96 h, were also significantly less ($p \leq 0.05$) than those of the corresponding control groups.

The RCCs of control and treated groups remained within the normal range for the first 36 h. In all subsequent groups, a highly significant decrease ($p \leq 0.01$) in RCC was noted for both groups (Figure 4.11b). Although the mean RCCs for treated groups after PZN injection were lower than those for the corresponding controls, the differences were not significant.

No significant changes were observed in the MCV values, either

within the control and the treated batch, or between corresponding groups of control and treated animals(Figure 4.11c).

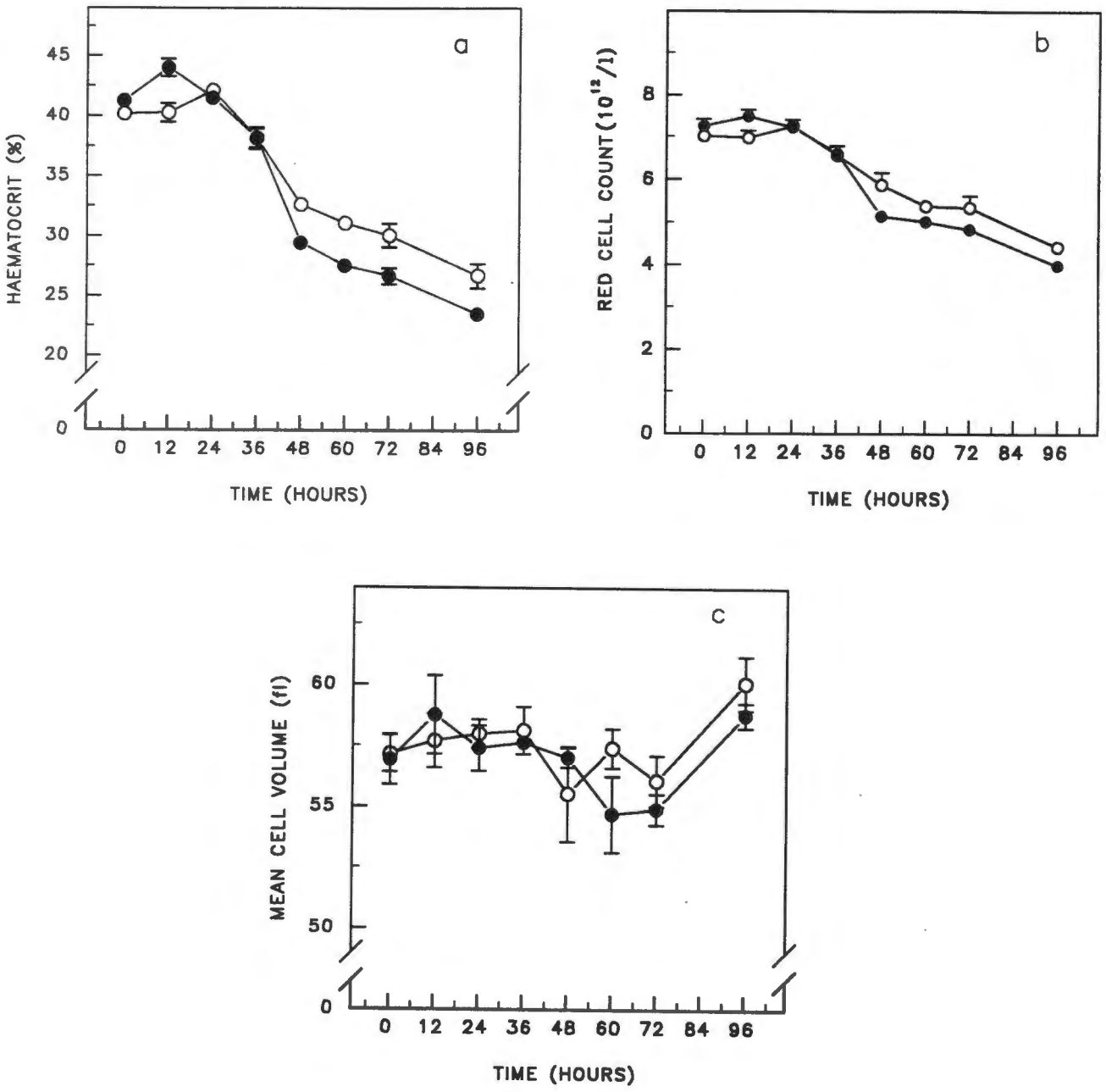


Figure 4.11. Haematological status of control(o) and treated (●) rats made anaemic with PZN after administration of saline and turpentine, respectively. a) Hct; b) RCC and c) MCV. The SEM of some data points are smaller than the circles.

4.6.3.2 ERYTHROPOIETIN

Serum Epo concentrations in both control and treated rats sacrificed at 12 h intervals, up to and including the 36 h interval, were within the normal range for Long-Evans rats (33·6 ± 5·8 mU/ml). Following the 36 h interval and after the injection of PZN, the Epo levels increased in both control and treated groups (Figure 4.12). The concentrations of the hormone measured in the serum of control and treated animals, sacrificed 48 h after initial treatment, were significantly higher ($p \leq 0.01$) than in those sacrificed immediately after the first injection (0 h groups). The peak Epo levels in control and treated rats were recorded in the groups sampled 60 h after saline or turpentine administration, i.e., 24 h after PZN injection. Epo levels in groups sacrificed at the end of the experiment (96 h groups) still had elevated serum levels of the hormone. At none of the intervals during the experiment were the Epo levels measured in treated animals significantly different from concentrations in corresponding controls.

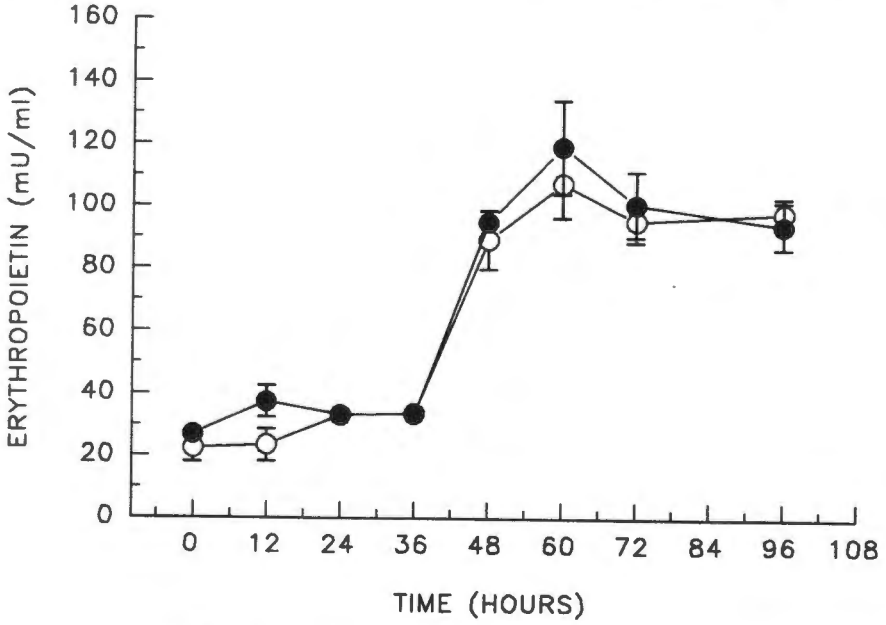


Figure 4.12. Serum Epo concentrations in groups of rats at different time intervals with (●) and without(○) acute inflammation. Anaemia was induced with PZN 36 h after initial treatment.

4.6.4 DISCUSSION

In this investigation, optimal Epo production during haemolytic anaemia in rats was analysed in the presence of an acute inflammatory reaction. The aim was to establish whether the mediators of acute inflammation could effect a blunted Epo response. Such a blunted reaction is believed to be a contributing factor to the persistent anaemia associated with chronic inflammation (Spivak *et al.*, 1989; Miller *et al.*, 1990). The macrophage-derived cytokines, IL-1 and TNFα, are regarded as the prime mediators of this effect (Jelkmann *et al.*, 1992; Faquin *et al.*, 1992). It has been demonstrated that these two cytokines, together with IL-6 and macrophage-conditioned medium, have the ability to down-regulate Epo expression in HepG2 cells *in vitro* (Chapter 2). These cytokines are the primary regulators of the acute phase reaction (Mortensin *et al.*, 1988; Andus *et al.*, 1988; Castell *et al.*, 1989). A blunted Epo response may, therefore, also be a feature of acute inflammation.

It has been established thus far that an increase in serum Epo levels, signifying enhanced renal biosynthesis of the hormone, occurs during aseptic acute inflammation. This was in response to the mild anaemia which had developed. It was also observed that acute inflammation can prolong recovery from experimentally induced anaemia, even in the presence of raised serum levels of Epo. This could have been mediated by the acute phase cytokines. Interleukin-1 was shown to inhibit the action of Epo on erythroid precursors (Schooley *et al.*, 1987). The data generated in the two earlier experiments were, however, not sufficient to determine whether *in vivo* Epo production was affected by acute inflammation. The reason for not solving this issue in the previous experiment was because stimulated Epo synthesis, in response to PZN-induced anaemia, did not coincide with the release of cytokines into the systemic circulation. In this

experiment the two endogenous responses have been synchronized.

Haematological indices, including serum Epo levels, measured in groups of control and treated rats after saline or turpentine administration, but before PZN injection, did not differ statistically and were within the normal range for Long-Evans rats. However, 12 h after administering PZN, a decrease was noted in the Hcts and RCCs of both control and treated groups (Figures 4.11a and b). The MCVs in groups of control and experimental rats did not change during the course of the experiment (Figure 4.11c). Although significant decreases ($p \leq 0.05$) occurred in RCCs of all animals after PZN administration, the value of this index in control groups did not vary statistically from that of corresponding treated rats. The Hct levels in treated groups after PZN injection were significantly ($p \leq 0.05$) lower than those in corresponding control rat groups. This slight exaggeration of anaemia in treated animals probably resulted from the additive effect of acute inflammation on erythropoiesis. However, since the RCCs in groups of control and treated rats did not differ, and because the difference between their Hct values were so slight, the degree of anaemia in control and treated groups can be regarded as similar, although not identical.

Erythropoietin concentrations increased in rats of both control and treated groups subsequent to PZN injection (Figure 4.12). In both groups, the peak serum levels of the hormone were detected 24 h after inducing haematological stress. Increased transcription of the Epo gene and synthesis of the hormone must have occurred over this period. The serum Epo levels gradually declined in subsequent groups. Neither the peak levels of the hormone, nor the levels measured at any other time interval in corresponding control and treated groups were statistically different. The mean levels in treated groups were, however, slightly, although not significantly, elevated above those of

associated controls. Since the degree of anaemia in the two groups was similar, the observation that their Epo levels were not significantly different, suggests that Epo synthesis was not inhibited during acute inflammation. Erythropoietin is, therefore, not an acute phase reactant.

How would one explain the inhibition of Epo synthesis by IL-1β, IL-6, and TNFα observed in HepG2 cells? It is known that cytokines are very potent, exerting their activities at concentrations in the nanomolar and picomolar range (Dinarello, 1988). Moldawer *et al.* (1987), evaluated serum levels of IL-1 and TNFα in mice with various inflammatory conditions, including turpentine-induced abscess in which an acute phase protein response was documented. They were unable to detect measurable amounts of the cytokines in any of the disorders and concluded that an hepatic acute phase response can occur during inflammatory states without the appearance of either IL-1 and TNFα in the circulation. Their assay was probably not sensitive enough, but the study does demonstrate that an APR may be elicited by very low concentrations of these cytokines.

Inflammatory mediators during acute inflammation may, thus, be high enough to induce the synthesis of acute phase reactants in the liver (which is beneficial), but at a level too low for a pathological effect on Epo production. The APR is, afterall, considered to be a host-generated protective mechanism aimed at containment or destruction of the inflammatory stimulus, removal of damaged tissue, and repair of the affected organ. Inhibition of Epo synthesis can hardly be considered beneficial to an organism, since acute phase responses can be precipitated by injuries in which extensive blood loss may also occur (Kushner, 1982; Schreiber *et al.*, 1989).

CHAPTER FIVE

ERYTHROPOIESIS AND ERYTHROPOIETIN PRODUCTION DURING CHRONIC INFLAMMATION

5.1 INTRODUCTION

Chronic inflammatory diseases, such as cancer, rheumatoid arthritis (RA), and the acquired immunodeficiency syndrome (AIDS), are often accompanied by an anaemia, commonly referred to as the anaemia of chronic disorders (ACD) (Cartwright, 1966; Cartwright and Lee, 1971; Hansen, 1983). It is a mild to moderate, normochromic/normocytic anaemia, which is characterized by reduced serum iron and total iron-binding capacity, with normal or increased iron stores (Cartwright and Lee, 1971; Means and Krantz; 1992). Reticulocytes are also not increased appropriately for the degree of anaemia, indicating that this is principally an "underproduction" anaemia (Means and Krantz, 1992).

Although frequently diagnosed, the pathogenesis of ACD has remained unclear. At least four pathologic processes are postulated to be involved (Cartwright, 1966; Pavlovic-Kentera *et al.*, 1979; Baer *et al.*, 1987). These are : a shortened erythrocyte survival, failure of the bone marrow to increase red blood cell production to compensate for an increased demand, impaired release of iron from the reticuloendothelial system (RES), and insufficient Epo production.

A shortened red cell life span has been reported in only a minority of patients (Lewis and Porter, 1960; Morgan and Jacox,

1964). In ten patients with different types of chronic inflammatory disease, Cavill *et al.* (1977), using a ferrokinetic technique, found mean red cell life spans in the range of 43 to 122 days. Al-Ismail *et al.* (1979), using the same technique, found values of 60 to 154 days in patients with Hodgkins disease. These results did not differ significantly from the range of 70 to 123 days obtained by this method in normal human adults. A shortened red cell survival, therefore, does not seem to be an important contributor to ACD.

The importance of iron metabolism in the anaemia of chronic disease has also been controversial (Means and Krantz, 1992; Cavill *et al.*, 1977; Douglas and Adamson, 1975). There is overwhelming evidence that the sequestration of iron in the reticuloendothelial system (RES) is an epiphenomenon unrelated to the anaemia (Williams *et al.*, 1982; Lukens *et al.*, 1967; Cavill and Bentley, 1982). The diversion of red blood cell iron to the RES cannot explain the anaemia in chronic disease, because the anaemia does not respond to oral iron (Davidson *et al.*, 1984; Ward *et al.*, 1969; Weber *et al.*, 1988). Orally administered iron was found to be absorbed normally in the anaemia secondary to uraemia (Weber *et al.*, 1988; Milman and Larsen, 1976) and normally, or almost normally, in the anaemia secondary to rheumatoid arthritis (RA). In comparison to normal controls, it is sometimes slightly decreased (Weber *et al.*, 1988), sometimes unchanged (Vreugdenhil *et al.*, 1990), and sometimes slightly increased (Boddyk and Will, 1969). Iron absorbed from the gastrointestinal tract binds to transferrin and transferrin-bound iron is incorporated efficiently into the erythron in the ACD (Finch *et al.*, 1970). Abnormal iron metabolism in chronic inflammation may, therefore, be of secondary importance in the pathogenesis of the associated anaemia.

Another mechanism proposed to explain the anaemia in chronic

disease, is the direct cellular or humoral inhibition of erythropoiesis resulting in the failure of the bone marrow to increase RBC production. Improved understanding of the pathogenesis of inflammation has led to the identification of cytokines that are involved in this process. Tumour necrosis factor-*alpha* levels have been reported to be increased in patients with cancer (Balkwill *et al.*, 1987), RA (Teppo and Maury, 1987), parasitic and bacterial infections (Teppo and Maury 1987; Kern *et al.*, 1989), as well as patients infected with HIV (Ayehunie *et al.*, 1993). Chronic administration of TNFα to animals resulted in the development of anaemia (Moldawer *et al.*, 1989; Tracey *et al.*, 1988) which was characterized by low serum iron and normal iron stores similar to ACD in humans (Alvarez-Hernandez *et al.*, 1989). When recombinant TNFα was administered to cancer patients in a phase I trial, those patients who completed four weeks of therapy became anaemic (Blick *et al.*, 1987).

Interleukin-1 is another cytokine whose levels are elevated in patients with RA, as well as other ACD associated conditions (Fujiwara *et al.*, 1986; Maury *et al.*, 1988), and this elevation correlates with markers of disease activity, such as anaemia (Maury *et al.*, 1988; Eastgate *et al.*, 1988). Interleukin-1 has been shown to inhibit murine erythropoiesis *in vitro* (Schooley *et al.*, 1987) and *in vivo* (Johnson *et al.*, 1989). In humans, decreases in haemoglobin in patients with RA were found to correlate directly with the IL-1 level (Eastgate *et al.*, 1988).

Both cytokines reduce erythropoiesis by inhibiting the proliferation of BFU-E and CFU-E cells (Roodman *et al.*, 1987; Maury *et al.*, 1988). These bone marrow cells are also the primary targets of Epo (Landschultz *et al.*, 1989; Dessypris *et al.*, 1988). The cytokines may, therefore, induce anaemia by antagonizing the biological effects of Epo. This may explain why exogenously administered Epo can resolve the anaemia in

chronic disease (Means *et al.*, 1989). Failure of the bone marrow to respond to endogenous increases in Epo levels, is considered by many to be the primary cause of ACD (Means and Krantz, 1992).

The supply of Epo to the bone marrow may also be the rate-limiting factor in the impaired marrow response in ACD (Pavlovic-Kentera *et al.*, 1979; Ward *et al.*, 1969). When Epo levels in patients with ACD were compared with those in patients with iron deficiency anaemia, they were found to be inappropriately low for the same degree of anaemia (Baer *et al.*, 1987; Spivak *et al.*, 1989; Miller *et al.*, 1990). This suggested that Epo synthesis may have been inhibited by humoral factors elaborated during the inflammatory process. Other investigators found Epo concentrations in the serum of patients with ACD to be normal (Douglas and Adamson, 1975; Birgegard *et al.*, 1987). However, the observation that inflammatory cytokines can suppress Epo synthesis *in vitro* (Chapter 2) lends support to the occurrence of a blunted Epo response in ACD. This finding prompted the current investigation into the synthesis of Epo in an animal model of chronic inflammation. Since the anaemia associated with RA often serves as a model for ACD, it was decided to study Epo production in a rat model of this chronic disorder.

5.2 DEVELOPMENT OF AN ANIMAL MODEL FOR THE ANAEMIA OF CHRONIC DISORDERS IN MAN. I.

Adjuvant-induced arthritis (AIA) has been widely used as a model for the study of human RA in view of their similar histopathological features. It is a chronic inflammatory polyarthritis that can be induced in rats by a single intradermal injection of an oil suspension of certain types of

heat-killed mycobacteria (Pearson, 1956; Pearson *et al.*, 1961). This preparation is usually referred to as complete Freund's adjuvant (CFA). Adjuvant-induced arthritis usually appears within 10 to 14 days after injection and is most prominent in the small joints of the extremities. Histopathologically, like RA in humans, it is characterized by polymorphonuclear and mononuclear infiltration of the synovium, synovial hyperplasia, pannus formation, destruction of cartilage, and bone erosion, progressing to ankylosis and deformities of the paws (Pearson, 1956; Pearson *et al.*, 1961; Van Vollenhoven *et al.*, 1988). Not all rat strains develop arthritis after inoculation with CFA (Battisto *et al.*, 1982; Stuart *et al.*, 1984). Several strains were, therefore, tested for susceptability to the disease.

5.2.1 MATERIALS AND METHODS

5.2.1.1 ANIMALS

Groups of three to five rats (weighing between 220 and 270 g), of the following strains were used: GH (Genetic hypertensive), LE (Long-Evans), LOU/C, SD (Sprague-Dawley), and WAG rats. The animals were kept under the same conditions as described in section 4.2.1.

5.2.1.2 INDUCTION OF ARTHRITIS

Each rat received a single intradermal injection (0.1 ml) in the footpad of the left hind paw of a preparation consisting of heat-killed *Mycobacteria tuberculosis* suspended in liquid paraffin (6 mg/ml). The suspension was sterilized by autoclaving (121 °C for 15 min) prior to administration. The magnitude of the inflammatory response was evaluated by

measuring the volume of the injected and uninjected (contralateral) hind paws to a level just above the insertion of the Achilles tendon into the gastrocnemius muscle. Paw volumes were measured with a mercury displacement apparatus (Figure 5.1). A rat was considered arthritic if an increase was noted in the volume of its contralateral paw within 20 days subsequent to adjuvant administration. An animal with swellings in its fore paws or tail was also regarded as having arthritis.

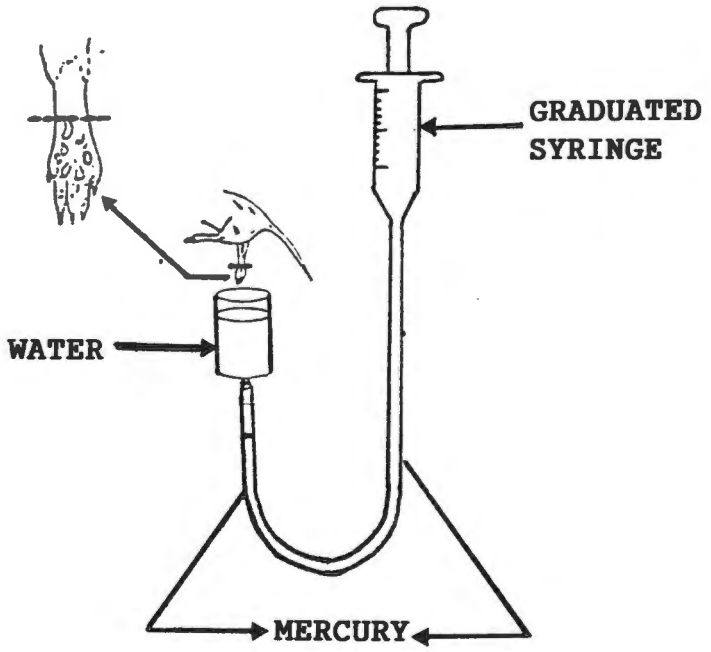


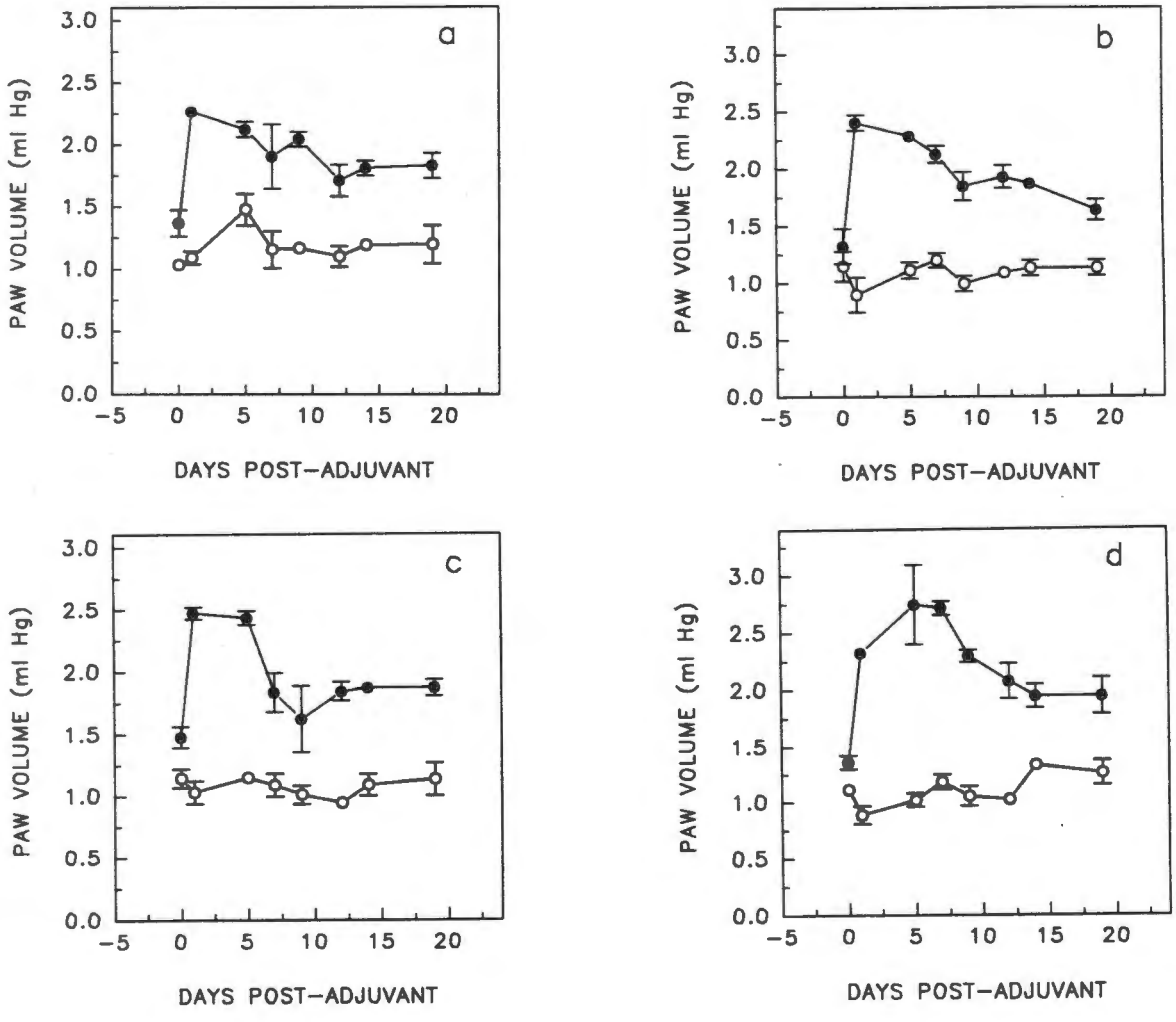
Figure 5.1. Mercury displacement apparatus used for measuring paw volumes.

5.2.1.3 STATISTICS

Data are expressed as means ± SEM. The student *t*-test was used to determine differences in volumes between the antigen-injected paws and uninjected paws. A *p* value less or equal to 0.05 was considered significant.

5.2.2 RESULTS

A single intradermal injection of CFA resulted in an acute inflammatory reaction in the injected paw of all rats within 12 to 24 h (Figure 5.2). By day 5, the initial swelling had subsided in the WAG, LE, and GH strains, whereas in the LOU/C strain it was less by day 7, and in the SD rats, by day 9. In WAG rats (Figure 5.2e) the volume of the injected paw started to increase gradually from day 12 until day 19 when the experiment was terminated. In the other rat strains, the oedema started to plateau off by day 12. The volumes of the injected paws, however, remained significantly higher ($p \leq 0.05$) than those of the uninjected paws of all rats up to day 19. None of the rats developed inflammation in their contralateral hind paw, their front limbs, or in their tail.



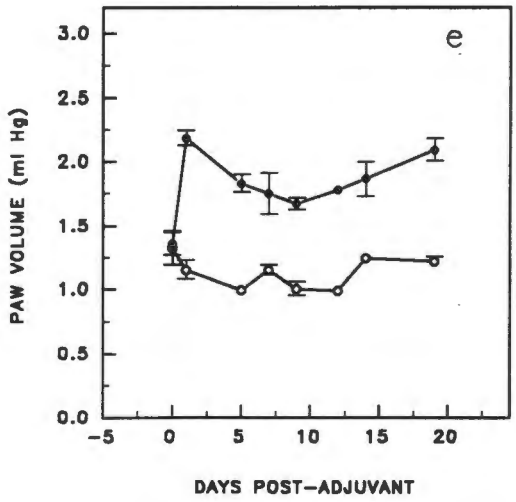


Figure 5.2. Volumes of the injected (●) and uninjected (○) hind paws of five different rat strains on several days following adjuvant administration. Paw volumes are expressed as the volume of mercury (Hg) displacement. a) GH rats, b) LE rats, c) LOU/C rats, d) SD rats, and e) WAG rats.

5.2.3 DISCUSSION

Five different rat strains were tested for susceptability to adjuvant-induced arthritis. None of the animals, all males, developed a polyarthritis characteristic of the disease. An acute inflammation, which lasted for 5 to 7 days, was induced in the injected paws of all rats by the CFA. Although the initial inflammatory response subsided, significant ($p \leq 0.5$) oedema still remained in the injected paw of each animal. This subacute inflammation persisted until the experiment was stopped. WAG rats showed signs of a secondary inflammatory reaction since their paw volumes started to increase steadily from day 12 (Figure 5.2e). In spite of the active inflammation in the injected paw of the rats, none of them developed arthritic lesions in their contralateral paw, front paws, or tail. This suggested that the five rat strains were either not genetically susceptible to AIA, or that the CFA was not

effective in inducing the disease.

Inoculating rats with CFA is not the only method for inducing arthritis. Another method is to immunize rats with collagen type II (Trentham *et al.*, 1977). Greater success has been achieved when rats were injected with a mixture of the two antigen preparations (Iisuka and Chong, 1982). The experiment was, thus, repeated but with a preparation consisting of both collagen type II and CFA. Only SD rats were used since CIA was successfully effected in this strain (Trentham *et al.*, 1977).

5.3 DEVELOPMENT OF AN ANIMAL MODEL FOR THE ANAEMIA OF CHRONIC DISORDERS IN MAN. II.

As mentioned above, another experimental model of arthritis, developed by Trentham *et al.* (1977), is collagen type II autoimmunity. They found that immunizing rats with purified native type II collagen resulted in the development of polyarthritis. Immunization via an identical protocol but with types I and III collagens or denatured type II collagen was not effective. The arthritis became evident only after an interval of latency and was associated with high levels of both cellular and humoral immunity to collagen (Trentham *et al.*, 1977; Stuart *et al.*, 1984). Types I and III collagens are found in the skin and parenchyma of several organs, whereas type II collagen exists in cartilage (Trentham *et al.*, 1977).

It has been demonstrated that AIA by mycobacteria and CIA are mediated by different pathogenetic mechanisms (Stuart *et al.*, 1984; Phadke *et al.*, 1984). It has also been reported that the pathogenetic mechanisms of AIA and CIA synergise to produce an extraordinary severe arthritic process (Iisuka and Chang, 1982). Since the incidence of both forms of experimental arthritis is

approximately 40% (Taurog *et al.*, 1985), it was decided to induce arthritis in rats with a preparation containing both collagen type II and *Mycobacterium butyricum*. Although AIA can be induced by several strains of mycobacteria, most studies were done with *M. butyricum* (Lukens *et al.*, 1967; Battisto *et al.*, 1982)

Experimental arthritis, whether adjuvant- or collagen-induced, occurs only in genetically susceptible rat strains. Both responsiveness (the magnitude of the immune reaction) and susceptibility (to the development of arthritis) after immunization are controlled by genes within, or linked to, the major histocompatibility complex (Battisto *et al.*, 1982; Stuart *et al.*, 1984). Sprague-Dawley (SD) rats have been shown to be highly susceptible (Trentham *et al.*, 1977; Ackerman *et al.*, 1979).

5.3.1 MATERIALS AND METHODS

5.3.1.1 ANIMALS

Forty male and forty female SD rats, weighing between 180 to 250 g, housed in pairs, were used. The animals were kept under the same conditions, and treated in the same manner, as described in section 4.2.1.

5.3.1.2 INDUCTION OF ARTHRITIS

Bovine type II collagen (Sigma, MO) was suspended in 0.1 M acetic acid and solubilized by the addition of pepsin (Sigma) in a ratio of 1:10 of collagen. The mixture was stirred overnight at 4°C. One part of collagen solution was emulsified with one

part of CFA containing *M. butyricum* (20 mg/ml). Each rat was injected intradermally in the plantar region of the left hind foot with 100 µl of the antigen preparation which contained 80 mg of collagen and 2 mg of *M. butyricum*. Fourteen days later the rats received a booster injection with an intradermal dose of 0.5 ml of the same preparation at four to five sites in the nape of the neck.

5.3.1.3 ASSESSMENT OF ARTHRITIS

Rats were inspected daily for the presence of distal joint swelling and erythema. A rat was considered arthritic if swelling and erythema were observed either in the tail or in at least one paw other than the injected foot. Arthritic lesions were photographed and radiographed.

5.3.1.4 HAEMATOLOGIC AND BIOCHEMICAL MEASUREMENTS

Haematological indices (Hct, Hb, RBC, WCC, MCV, MCH, and MCHC) were determined as described before (section 4.2.3). Erythropoietin concentrations in serum were assayed with a RIA kit (Incstar) described in section 4.2.7.

Serum iron levels were quantified with a commercially available kit (Boehringer Mannheim) based on an automated micromethod (Siedel *et al.*, 1984; Brozovic and Purcell, 1974). The method does not require deproteinization of the serum sample. In the test, ferric ions (Fe^{3+}) are separated from transferrin by means of guanidinium chloride, at a weakly acidic pH, and reduced to ferrous ions (Fe^{2+}) with ascorbic acid. The Fe^{2+} ions then form a coloured complex with FerroZine (a patented chromogen). The intensity of the coloured complex, which is a measure of the

concentration of iron in serum, is read with an autoanalyzer (BM/Hitachi System 704).

5.3.1.5 STATISTICAL ANALYSIS

Data are expressed as means ± SEM. One-way analysis of variance was used to determine (least) significant differences in laboratory measurements between normal control rats and those with experimental arthritis. A *p* value less or equal to 0.05 was considered significant.

5.3.2 RESULTS

5.3.2.1 DEVELOPMENT OF ARTHRITIS

The incidence of arthritis following immunization with the antigen preparation was very low. Only five, all females, out of a total of eighty rats developed the condition which appeared more than two months after the footpad inoculation. Arthritic lesions were observed in the joints of one or both front paws, in the untreated hind paw, and in some rats, even in the tail (Figures 5.3 and 5.4). Roentgenographs showed significant bone destruction at the sites of inflammation.

a

b

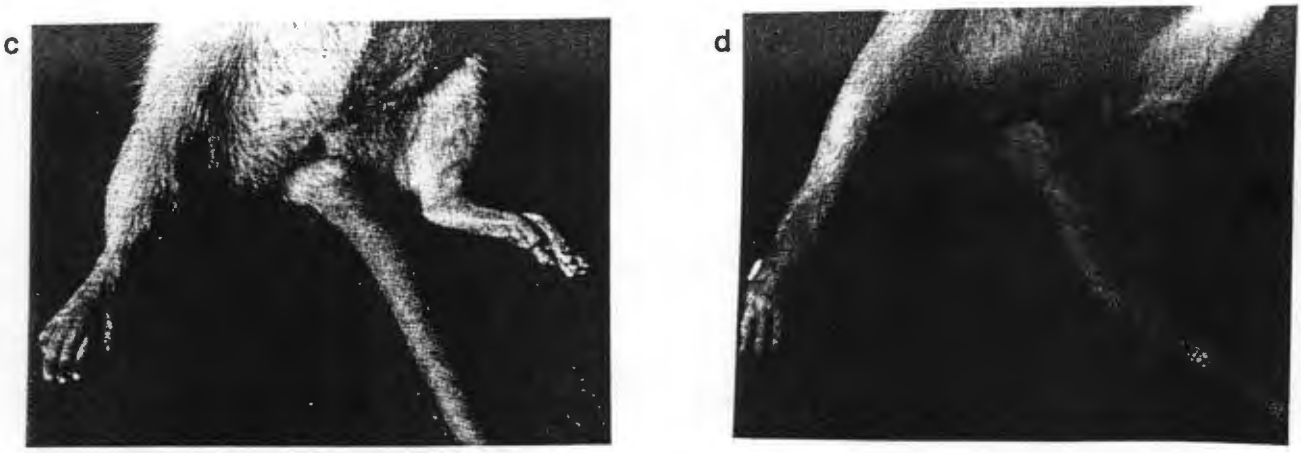


Figure 5.3 Joint swelling in rats with antigen-induced arthritis. a) Normal front paws. b) Front paw in rat with arthritis. c) Normal hind limbs. d) Hind limbs in arthritic rat.

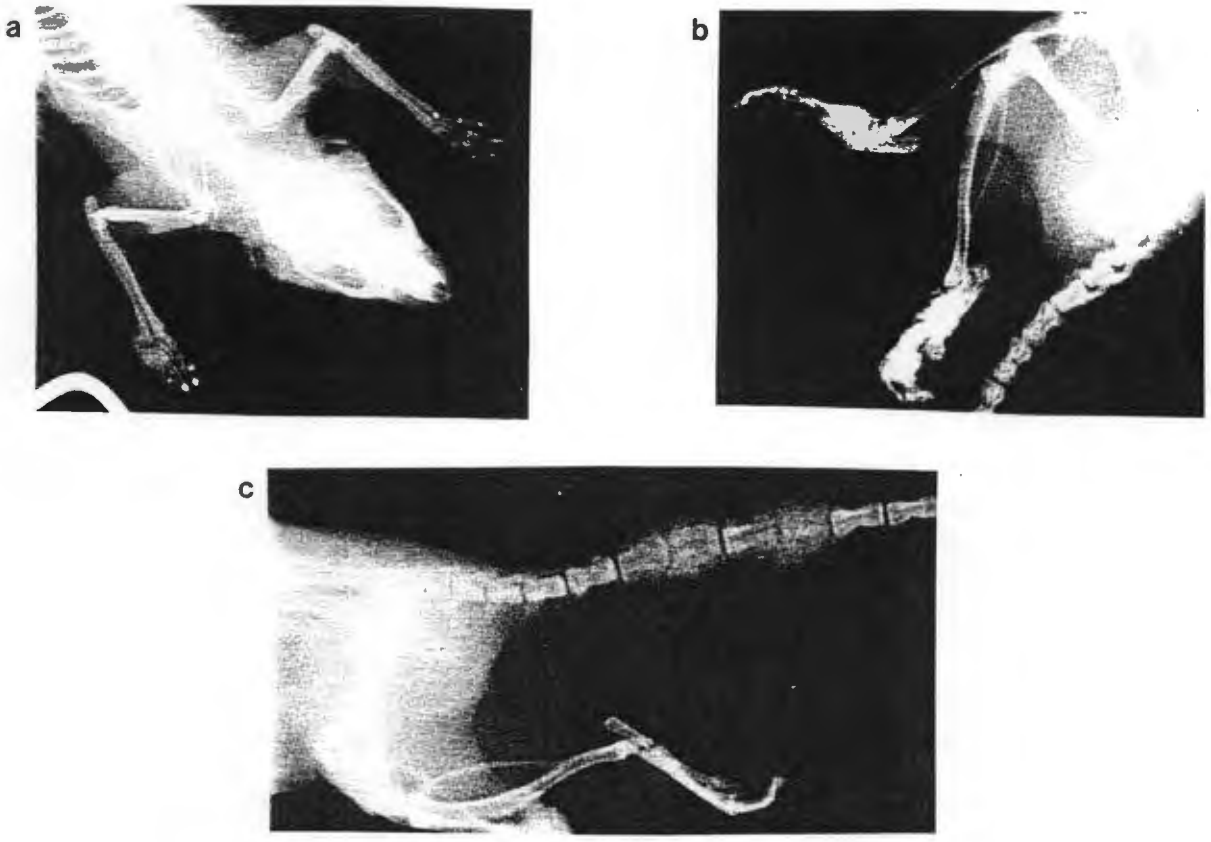


Figure 5.4 Roentgenographs of rats with arthritis. a) Normal left front limb with arthritic joint in right limb. b) Both hind paws are inflamed. The one nearest to the tail was injected with the antigen preparation. c) Arthritic lesion in the tail. Note the normal appearance of the hind paw.

5.3.2.2 HAEMATOLOGY

None of the animals with arthritis were found to be anaemic. Values for the different haematologic parameters were well within normal limits for female rats of this strain (Table 5.1). The WCCs in two rats were significantly higher than normal whilst the serum iron in one of these rats was well below that recorded for normal controls. These are signs of the inflammatory process.

NORMAL CONTROLS	Hb g/dl	Hct %	RCC 10^{12}/l	WCC 10^{9}/l	MCV fl	MCH pg	MCHC g/dl	SERUM IRON µmol/l	Epo mU/ml
(n=10)	14.79 ± 0.115	39.51 ± 0.355	7.10 ± 0.052	5.69 ± 0.42	55.6 ± 0.265	20.89 ± 0.175	37.52 ± 0.346	47.2 ± 3.454	31.27 ± 2.881
RATS WITH ARTHRITIS	14.5	43.6	7.48	6.3	58.2	19.4	33.4	56.3	31.0
	13.3	39.1	7.01	12.1*	55.8	19.0	34.0	27.4☎	31.0
	15.4	44.1	8.02	9.4*	54.9	19.2	34.9	44.5	39.0
	14.2	40.3	7.13	2.9	56.5	19.9	35.1	ND	25.0
	14.2	42.0	7.26	2.6	57.8	19.6	33.9	73.8	21.0

ND=NOT DETERMINED

TABLE 5.1 Haemopoietic indices, serum iron, and serum Epo levels in five female Sprague-Dawley rats with antigen-induced arthritis. The mean values with their SEM for each parameter found in ten normal rats are given in the top half of the table. The WCCs in two rats (*) were elevated above the normal value, indicating pronounced disease activity. One of these rats (☎) also had a reduced serum iron level. None of the rats was, however, anaemic.

5.3.4. DISCUSSION

Adjuvant- or collagen-induced arthritis in rats are widely accepted models for the study of human rheumatoid arthritis (Pearson, 1956; Pearson *et al.*, 1961; Van Vollenhoven *et al.*, 1988). Since the anaemia in RA patients has often served as a model for ACD, it was decided to use the arthritic rat model for investigating Epo biosynthesis during chronic inflammation.

Arthritis was induced in SD rats with a water in oil emulsion containing heat-killed *M. butyricum* and solubilized bovine type II collagen. A booster injection was also administered in order to elicit an enhanced secondary immune response to the antigen preparation. In spite of this, the incidence of the condition was only 6 percent, with the disease appearing more than two months after the initial immunization. The rats which developed the condition were all females, suggesting that male SD rats may not be as susceptible to AIA. The affected animals presented with swellings in the joints of one or more limbs, and some even had lesions in the tail. Roentgenographs demonstrated significant bone destruction and new bone formation at the sites of inflammation.

Although the inflammatory lesions were severe, none of the animals presented with signs of anaemia (Table 5.1). The measured (Hct, Hb, and RCC) and calculated (MCV, MCH, and MCHC) haematological parameters for all diseased rats did not differ statistically from those of normal controls. These results suggest that anaemia does not always accompany experimentally induced arthritis in rats. This is not unusual, since not all patients with RA are anaemic (Birgegard *et al.*, 1987; Reid *et al.*, 1984). Antigen-induced arthritis in rats, although an excellent model for studying the pathology of RA in humans, is, therefore, not ideal as a model for ACD. A model for an

alternative chronic disorder which is also associated with anaemia was, thus, required. Since cancer patients are also frequently anaemic, (Bentley, 1982), an animal model for this disease was used.

5.4 ERYTHROPOIETIN SYNTHESIS IN A MURINE CANCER MODEL

Anaemia in cancer patients, which is not due to marrow invasion by tumour or marrow suppression by chemotherapy, is also classified as ACD (Hansen, 1983). A number of animal cancer models are reported in the literature (Fidler, 1978; Hart, 1979; Zucker *et al.*, 1979; MacManus *et al.*, 1990). Cancer is usually induced by the transplantation of tumour tissue (Fidler, 1978), or by the injection of cancer cells (Hart, 1979; Zucker *et al*, 1979), into a susceptible host. Solid tumours usually arise after seven days at the site of transplantation or injection. A profound anaemia is also in evidence at this stage (Zucker *et al.*, 1979; MacManus *et al.*, 1990).

In this investigation, cancer was induced in C57BL/6J mice with a suspension of B16-F10-BL6 melanoma cells. The model was described by Fidler (1978) and Hart (1979) who characterized it in terms of the growth rate of the tumour, its dissemination and tissue invasiveness, as well as the inhibitory and enhancing responses of the host.

5.4.1 MATERIALS AND METHODS

5.4.1.1 ANIMALS

The experiments were performed using inbred C57BL/6J mice which were originally obtained from Jackson Laboratories (Bar

Harbour, Maine, USA). A breeding colony was developed, and has since been maintained at the National Institute for Nutritional Diseases (Tygerberg, SA).

Male mice, weighing between 20 to 25 g, were housed in groups of five to eight in polypropylene cages with metal rung tops, and kept in a well-ventilated room with a 12 h day/night cycle and ambient temperature of 23 ± 1°C. The animals had free access to clean water and rodent chow.

5.4.1.2 CELLS

B16-F10-BL6 mouse melanoma cells were provided by Dr C F Albrecht (Department of Pharmacology, Stellenbosch University, Tygerberg, SA). The cells were originally obtained from Dr I J Fidler of the Cancer Metastasis and Treatment Laboratory (National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland, 21701). The cells were cultured in McCoy's SA medium supplemented with 10% heat-inactivated FCS, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

5.4.1.3 TUMOUR SYSTEM

B16-F10-BL6 melanoma cells were harvested from nonconfluent monolayers by a 2 min incubation with a solution of 0.25% trypsin and 0.02% EDTA. The cells were washed once and the suspension adjusted to 2 x 10^6 cells/ml of culture medium. Viability was assessed by trypan blue exclusion, and only suspensions containing ≥95% viable cells were used. Mice were injected with 1 ml (i.p.) of the tumour cell suspension.

Control mice were injected with culture medium. All treatments were done under aseptic conditions. Starting from day ten after inoculation, blood was drawn via cardiac puncture from five to seven mice per day. The animals were anaesthetized with a solution containing 100 mg ketamine and 5 mg xylazine/ml. Each mouse received an i.p. dose of 5 µl/g. After cardiac puncture, the mice were sacrificed by cervical dislocation. Blood was collected into microtainer tubes containing EDTA pellets (Radem laboratories). The following organs were collected from two cancer and two control mice for histological studies: Spleen, kidney, liver, testis, bladder, heart, lung, brain, intestine, and bone marrow. Organs were stored in 10% formalin until such time that the histological studies could be carried out.

5.4.1.4 EXPERIMENTAL ANAEMIA

A second control group with experimental anaemia was required to compare their Epo response with that of mice with cancer. A group of twenty mice were injected (i.p.) with sterile phenylhydrazine hydrochloride (60 mg/kg) in isotonic saline. Blood was sampled via cardiac puncture from groups of five animals on subsequent days in order to obtain haematological parameters for mice ranging from very, to mildly anaemic. A second group of five mice received a dose of phenylhydrazine on two consecutive days. Blood was collected from this group, which had severe anaemia, on the day following administration of the second dose.

5.4.1.5 HAEMATOLOGIC AND BIOCHEMICAL DETERMINATIONS

Haematological parameters (Hb, Hct, RBC, WCC, MCV, MCH, and MCHC) were determined with a Coulter counter as before (section 4.2.3). Serum was prepared from whole blood as described in

section 4.2.4. Serum Epo concentrations were measured with an RIA kit (Incstar) as outlined in section 4.2.7, and serum TNFα levels with an ELISA kit (Genzyme) described in section 4.2.8.

5.4.1.6 STATISTICAL ANALYSIS

Data are represented as means ± SEM. Comparisons between control mice and those with cancer were carried out using ANOVA. A *p* value less or equal to 0.05 was considered significant.

5.4.2 RESULTS

5.4.2.1 TUMOUR DEVELOPMENT

All mice injected with melanoma cells developed tumours in their peritoneal cavities. The tumours gradually increased in size until the animals died; usually after 15 to 17 days following inoculation. No tumours were observed in control mice.

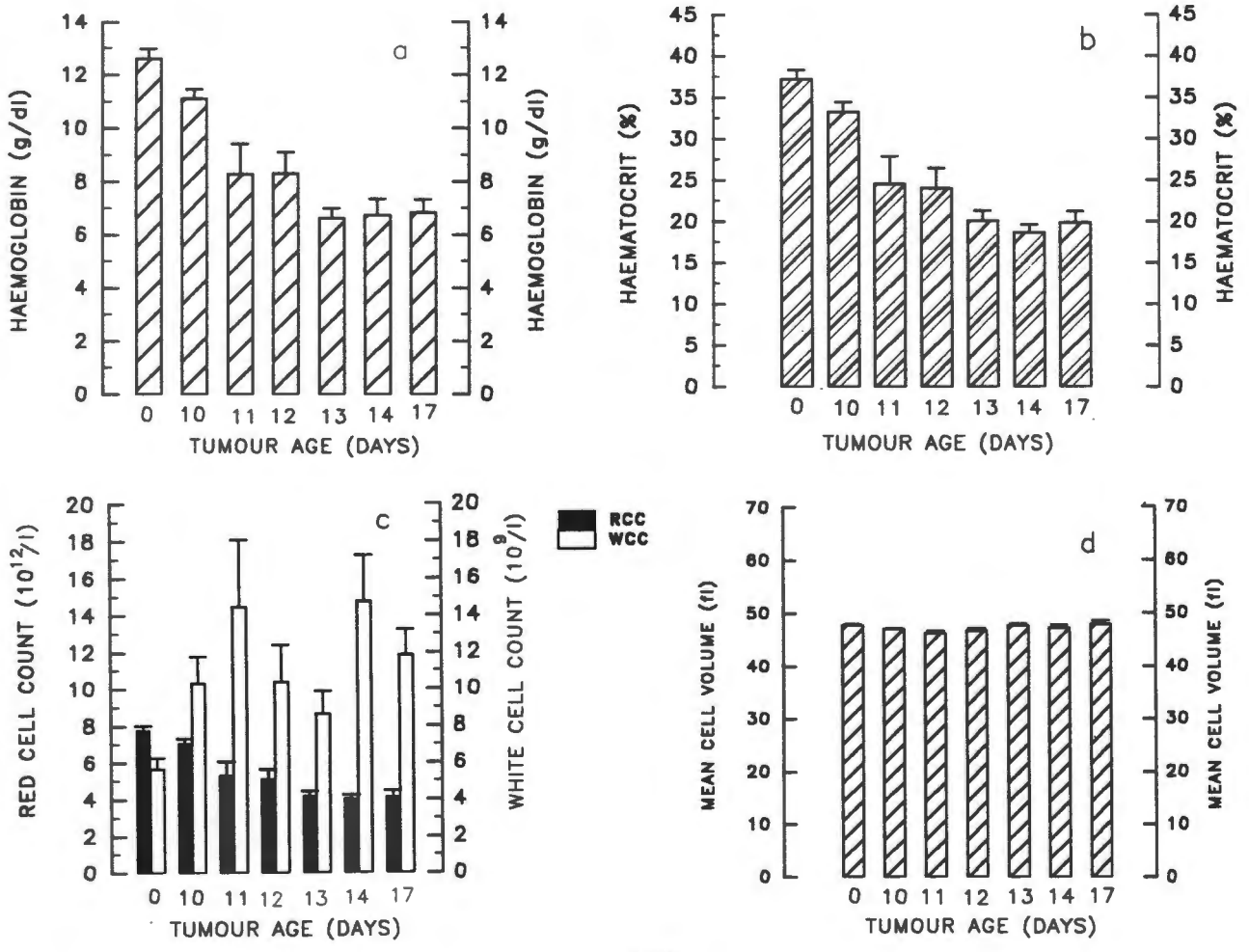
5.4.2.2 HISTOLOGY

The histological appearance of the kidneys, liver, testis, bladder, heart, intestine, and brain in mice with cancer did not differ from those in control animals without the disease. Pulmonary metastases occurred in the mice with cancer. Metastatic epithelial melanomas were found in the peribronchial tissue, as well as in the alveolar walls of the lung. Tumour growth was also accompanied by massive splenomegaly. Pronounced extramedullary haemopoiesis could be observed in the spleen. Intense erythropoiesis and megakaryocytopoiesis could be detected in the red pulp. Total displacement of the normal red

pulp by cellular elements of the bone marrow was found. The white pulp of the spleen was morphologically normal. The bone marrow was also extremely active with haemopoiesis. There was no evidence of marrow infiltration by tumour cells.

5.4.2.3 HAEMATOLOGICAL INDICES

A steady decline in values of erythropoietic parameters (Hb, Hct, and RCC) accompanied tumour growth (Figure 5.5). The normal Hb range for ten C57BL/6J mice was found to be 12.62 ± 2.28 g/dl (2 SD). Mice that survived for 14 days after cancer cell inoculation had a mean value as low as 6.74 ± 2.69 g/dl (n=5), indicating severe anaemia. A progressive leucocytosis was also present (Figure 5.5c). No changes occurred in the MCV, MCH, and MCHC values (Figure 5.5d-f).



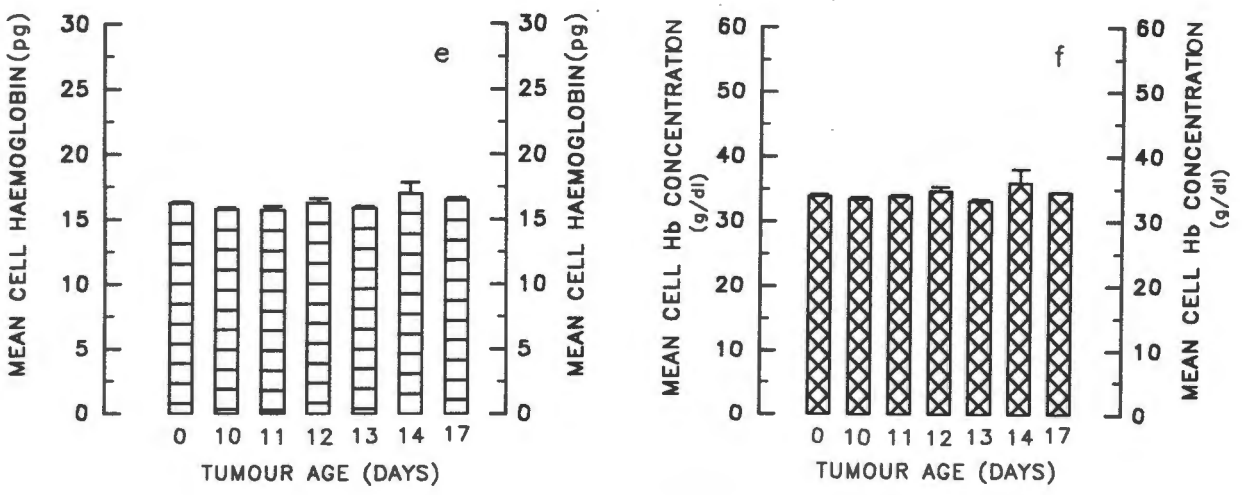


Figure 5.5 Haemopoietic parameters of mice with cancer at different stages of advancement. a) Hb, b) Hct, c) RCC and WCC, d) MCV, e) MCH, and f) MCHC.

5.4.2.4 SERUM ERYTHROPOIETIN

Figure 5.6 represents immunoreactive Epo concentrations in relation to Hb values for groups of mice with tumours at different stages. As the disease progressed and the animals became more anaemic, they responded by increasing their serum levels of the hormone. In Figure 5.7 serum Epo concentrations are plotted against Hct values for tumour-bearing mice and control mice with haemolytic anaemia. A negative correlation between the two parameters existed in both groups. The two correlation curves were not statistically different. When the mice were grouped according to their degree of anaemia, and their Epo levels compared, the most anaemic (Hct: 15 to 20 %) tumour-bearing group had Epo levels significantly lower ($p \leq 0.05$) than the control group with the same degree of anaemia (Figure 5.8). In all the other groups, the Epo levels of anaemic controls and mice with cancer were essentially the same. This suggested that Epo production was inhibited only in mice which were severely anaemic. This group included mice with tumours older than 14 days, i.e., the animals with advanced cancer (Figure 5.6).

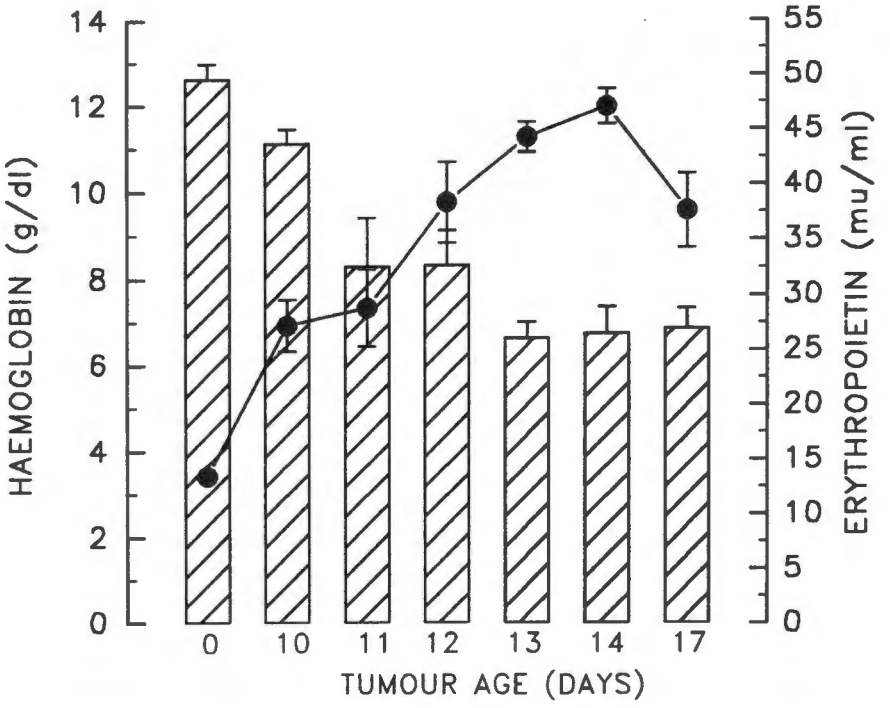


Figure 5.6 Serum Epo concentrations and Hb values of mice with cancer. Note how the Epo levels increased as the Hb concentrations declined with the progression of the disease. This relationship persisted until the fourteenth day. In mice with 17 day-old tumours, the Epo concentrations decreased without an improvement in the anaemia.

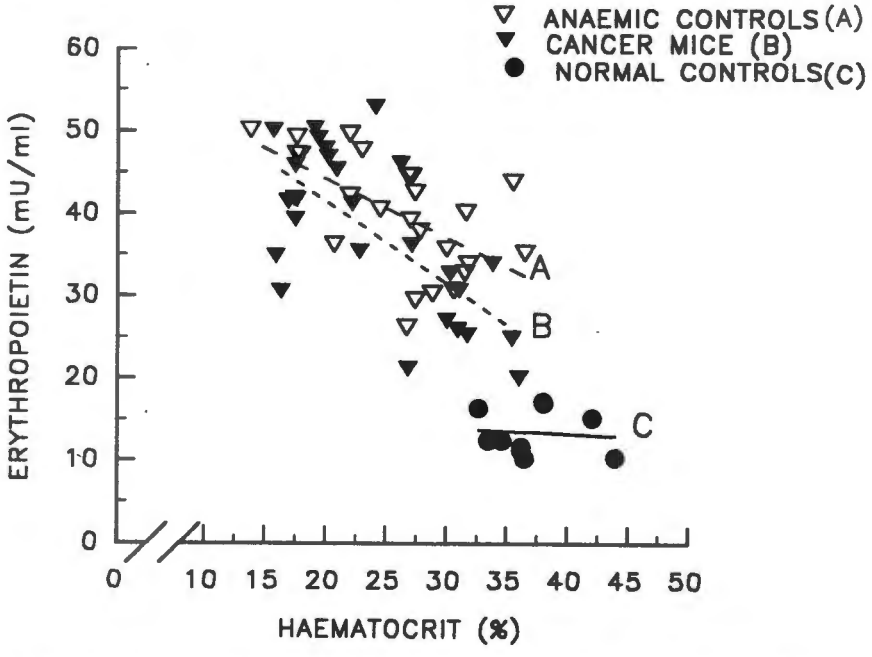


Figure 5.7 Regression lines between serum Epo concentrations and Hct values of mice with cancer and those with haemolytic anaemia. A negative correlation between the two parameters (which did not differ statistically) existed in both groups.

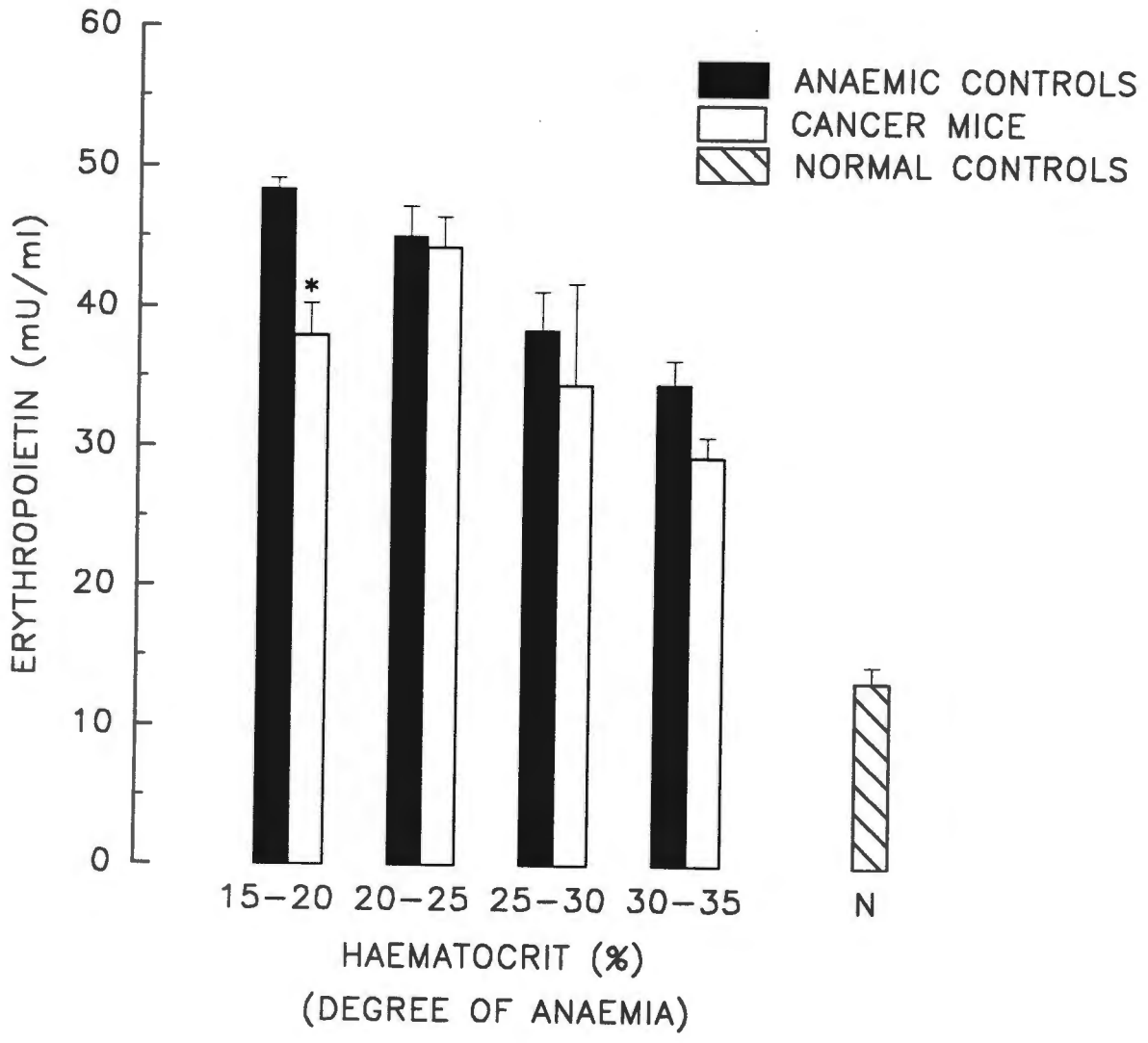


Figure 5.8 Serum Epo concentrations of tumour-bearing mice and controls, grouped in terms of their degree of anaemia. Only the most anaemic cancer group(*) had significantly lower levels of the hormone compared to controls.

5.4.2.5 TUMOUR NECROSIS FACTOR-*alpha*

Tumour necrosis factor-*alpha* levels did not correlate with the degree of anaemia (Figure 5.9). No correlation could be found between serum levels of this cytokine and any of the red cell indices. There was also no relationship between Epo concentrations in serum and that of TNFα. It is noteworthy, however, that the serum TNFα levels of mice with cancer exceeded those of rats with acute inflammation (Figure 5.10).

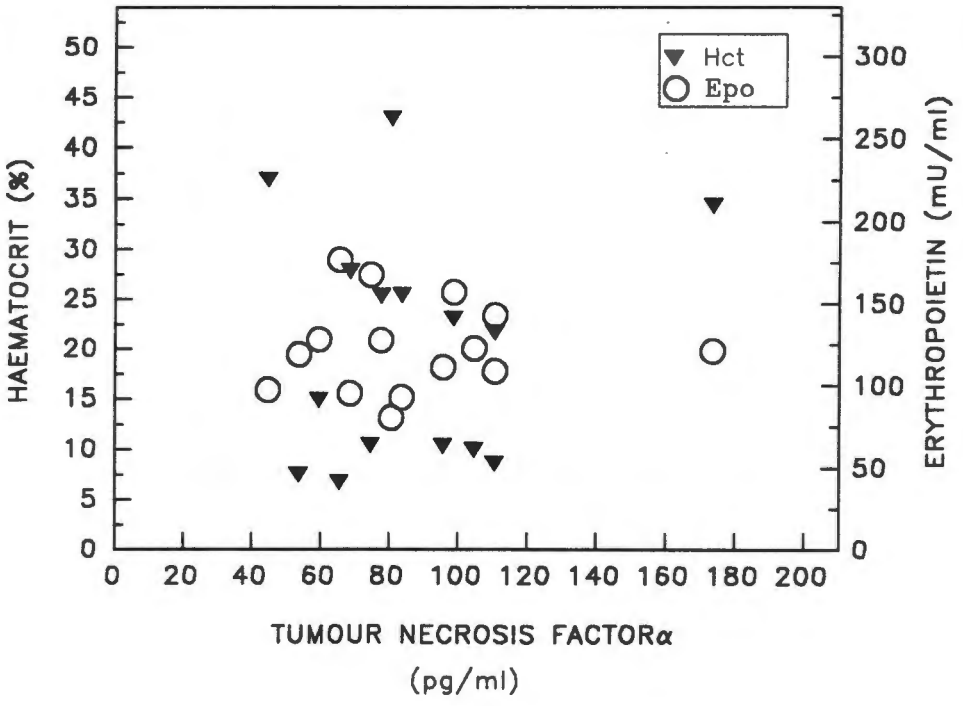


Figure 5.9 Serum TNFα levels versus Hct and Epo levels of mice with cancer. No correlation existed between TNFα concentrations and the other two parameters, indicating that the cytokine was not a true marker for the degree of anaemia or for disease activity.

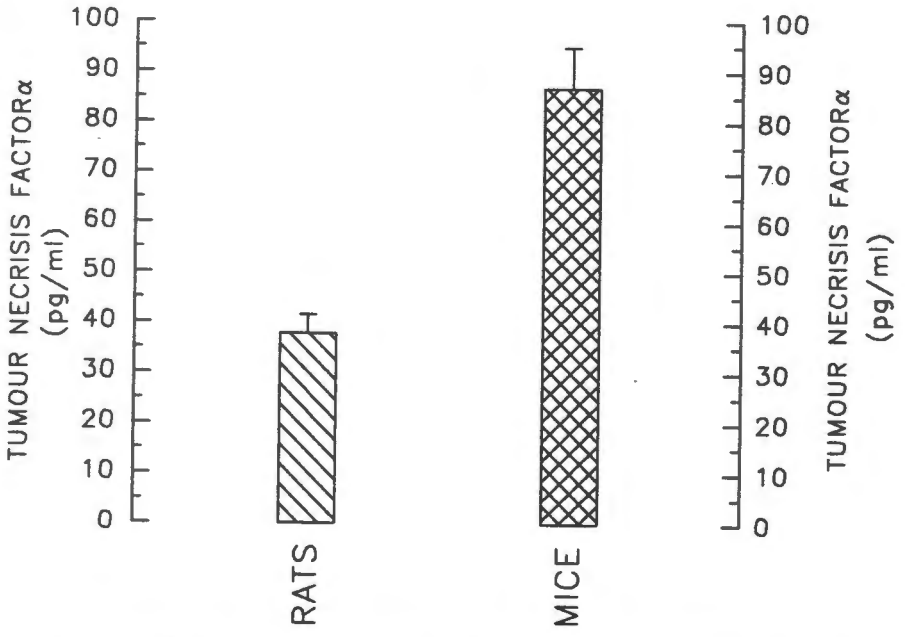


Figure 5.10 Serum TNFα concentrations in LE rats (n=6) with acute inflammation and C57BL/6J mice (n=15) with cancer. Acute inflammation was induced with a subcutaneous injection of turpentine (0.5 ml/100 g). Mice with cancer had significantly higher levels ($p \leq 0.05$) of the cytokine than rats.

5.4.4 DISCUSSION

In this investigation, erythropoiesis and erythropoietin biosynthesis were followed in mice with cancer of increasing severity, i.e., from the time they first showed signs of anaemia (tenth day) until they died (seventeenth day). The aim was to determine if Epo production was appropriate for the degree of the associated anaemia. This could be established by comparing the serum levels of the hormone in these animals with the levels measured in mice with a similar degree of haemolytic anaemia, in which the synthesis of the hormone was optimal.

The anaemia was found to be progressive, becoming worse as the disease progressed. Serum Epo concentrations also increased as the anaemia developed, until the fourteenth day. Thereafter, the levels declined. When the Epo levels in diseased mice were compared to those of controls with the same degree of anaemia, no statistical difference could be demonstrated (Figure 5.7). This suggested that, in general, the Epo response in mice with cancer was appropriate for the associated anaemia. This is in contrast to what was found by MacManus *et al.* (1990), who reported the absence of a correlation between the Hct and serum Epo concentrations in mice with a transplantable murine mammary carcinoma. Miller *et al.* (1990), on the other hand, detected elevated serum levels of the hormone in patients with cancer, but found a lower incremental increase in their Epo response compared to patients with iron deficiency anaemia.

When control and experimental mice were grouped in terms of the severity of their anaemia and their Epo concentrations compared (Figure 5.8), it was found that the most anaemic cancer group had significantly lower serum Epo concentrations than the corresponding control group. These mice were those with seventeen day-old tumours, i.e., the very sick animals. This

suggested that a blunted Epo response is dependent on disease activity. Whether the response was specific, however, seems questionable. It is more likely that the lower Epo response was representative of a generalized failure of the body's metabolism due to excessive disease activity. These animals suffered from cachexia and showed no interest in food or water. In fact, they did not have the strength to feed themselves. They were totally immobile and did not respond to physical stimuli. Under such conditions, the biosynthesis of any hormone would be negatively affected.

How would one then explain the specific inhibition effected by the inflammatory cytokines on *in vitro* Epo synthesis (Chapter 2)? The answer may lie with the cell line that was used. HepG2 cells are transformed liver cells (Knowles *et al.*, 1980). The liver, which is the site of acute phase protein synthesis (Kushner, 1982), appears to be extremely sensitive to cytokines. Moldawer *et al.*, (1987) observed changes in acute phase reactants in the absence of detectable amounts of inflammatory cytokines. Rats with turpentine-induced acute inflammation had reduced albumin but optimal Epo concentrations at the time when TNFα levels could be demonstrated in their serum (Chapter 4). These observations suggest that the kidney cells which manufacture Epo *in vivo* are not as susceptible to cytokine-mediated effects as normal hepatocytes or HepG2 cells. Whenever a cell-type becomes cancerous, it often reverts to the embryonic state. HepG2 cells, for example, secrete *alpha*-foetoprotein (Knowles *et al.*, 1980), a protein normally expressed during foetal life (Crandall *et al.*, 1989). Erythropoietin is synthesised by the liver in the foetus (Zanjani *et al.*, 1977) and mainly by the kidney after birth (Jacobson *et al.*, 1957; Adamson *et al.*, 1968; Bondurant and Koury, 1986). The reason for this switch is still obscure. A possible explanation, based on the findings of this investigation, might be to protect the organism, once born, from succumbing to anaemia during

disturbances in its homeostasis that may precipitate acute phase responses. This hypothesis can only be tested by isolating the renal cells which synthesise Epo and characterizing their cytokine receptors. To date, the identity of the specific cell in the kidney that produces Epo has remained a mystery.

CHAPTER SIX

CONCLUSION

The primary objective of this investigation was to determine whether a blunted erythropoietin response, hypothesized to be mediated by inflammatory cytokines, contributed to the anaemia of chronic disorders. Epo synthesis during acute inflammation was also studied, since the cytokines implicated in the blunted Epo response, are also the regulators of acute phase protein synthesis. The results obtained in this investigation, enabled the following conclusions to be made:

1. Macrophage-conditioned medium (MCM) and recombinant IL-1ß, IL-6, and TNFα can effect dose-dependent reductions in *in vitro* Epo synthesis by the hepatoma cell line, HepG2. Interleukin-1ß and TNFα appeared to mediate their effects by inhibiting transcription of the Epo gene, whereas IL-6 seemed to exert its action by modulating a post-transcriptional event in the Epo biosynthetic pathway. Interleukin-1ß and TNFα potentiated each others effects on Epo production. Potentiation was, however, not observed between IL-6 and the other two cytokines. All three cytokines synergized with MCM in reducing HepG2 Epo output.

2. A mild anaemia developed in rats with turpentine-induced acute inflammation. The reduced erythropoiesis elicited an elevation in their serum Epo concentrations. Prior to the increase, the serum Epo levels fluctuated in accordance with a circadian rhythm similar to those of normal controls. With the drop in haemopoiesis, however, the rhythm was broken as the serum Epo concentrations increased. The reduction in haematological values

coincided with an increase in serum TNFα concentrations. A decrease was also noted in the serum levels of albumin, a negative acute phase reactant. Inflammatory cytokines, however, had no effect on *in vivo* Epo production. Serum concentrations of the hormone in rats with acute inflammation and phenylhydrazine-induced anaemia did not differ statistically from those of control rats with the same anaemic profile but without acute inflammation. The acute inflammatory reaction did, however, prolong the duration of the phenylhydrazine-induced anaemia which resulted in continued high serum levels of Epo.

3. Adjuvant-induced arthritis in rats is a poor model for studying the anaemia of chronic disorders in man. The disease failed to develop in several rat strains, including GH, LE, LOU/C, and WAG . Even male Sprague-Dawley (SD) rats did not develop arthritis. Success was only achieved with female SD rats. The incidence was, however, very low (12% of all female rats). Although the affected rats developed several arthritic lesions, none of the animals became anaemic. Their serum Epo levels were within the normal range as well.

The mouse cancer model that was selected proved more successful. The mice (C57BL/6J) became anaemic within ten days of inoculating them with B16-F10-BL6 melanoma cells. Histological examination ruled out tumour invasion of the bone marrow as the cause of the anaemia. In fact, the bone marrow was extremely active with erythropoiesis, as was the spleen. The anaemia became worse as the cancer progressed. Serum Epo concentrations, however, increased in response to the developing anaemia. This increase was found to be appropriate for most part of the disease process since the correlation curve between Hct values and Epo levels for mice with cancer, did not differ significantly from that

of control mice with phenylhydrazine-induced anaemia. However, when the animals were grouped in terms of their degree of anaemia and their Epo concentrations compared, it was found that the most anaemic cancer group had a significantly lower Epo level than the corresponding anaemic control group. These mice were those with the most advanced melanomas, suggesting that a blunted Epo response was dependent on disease activity.

The data seem to suggest that a blunted Epo response does not contribute to anaemia of chronic disorders. It may become a contributing factor only in cases of severe cachexia, as in terminal cancer patients. Although cytokines like IL-1ß, TNFα, and perhaps IL-6 have the ability to inhibit Epo synthesis, such inhibition does not occur readily *in vivo*. It may be that the renal cells which produce Epo, unlike hepatocytes, do not have receptors for these cytokines. The cytokines, especially IL-1ß and TNFα, may nevertheless cause ACD, but by a mechanism of suppressed erythropoiesis in the bone marrow. They may act by antagonizing the action of Epo on Epo-sensitive erythropoietic precursors. Higher concentrations of Epo than that secreted by the kidney may, therefore, be required to overcome their inhibitory effects. It is postulated that renal Epo output is dependent on blood oxygen tension (Goldberg *et al.*, 1988,1989) and, thus, not on bone marrow requirements. This may explain why exogenous Epo has been so successful in correcting the anaemia of patients with rheumatoid arthritis (Pinctus *et al.*, 1990), cancer (Abels, 1992), and even AIDS (Fischl, 1990).

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APPENDIX

MEDIA AND SOLUTIONS

Acrylamide (40%)

Acrylamide		38 g
N,N'-methylenebisacrylamide		2 g
H_2O	to	100 ml

Acrylamide/Urea Solution

Acrylamide (40%)		15 ml
TBE (10X)		10 ml
Urea		48 g
H_2O	to	100 ml

Ammonuim Persulfate (50%)

Ammonium persulfate		0.5 g
H_2O	to	1 ml

Bromocresol Green Stock Solution (0.6 mmol/l)

Bromocresol green	419 mg
Sodium azide	100 mg
NaOH (0.1 M)	10 ml
Water, up to	1000 ml

Bromocresol green working solution was prepared by mixing one volume of stock solution with three volumes of succinate buffer (0.1 M) and adjusting the pH to 4.2.

Chromic Acid Solution

Chromium (VI) oxide	84 g
H_2O	700 ml
Sulphuric acid	400 ml

Column Loading Buffer (2X)*

Tris. Cl (pH 7.6)	40 mM
NaCl	1 M
EDTA	2 mM
Sodium lauryl sarcosinate (SLS)	0.2 %

* 1X column loading buffer was prepared by diluting 1 part of the 2X buffer with 1 part DEPC-treated sterile water.

Column Buffer (RNase protection)

NaCl	0.1 M
Tris (pH 7.5)	20 mM
EDTA	1 mM
SDS	0.1 %

Denhart's Solution (100X)

Polyvinylpyrolidine	1 g
Bovine serum albumin	1 g
Ficoll 400	1 g
H_2O	to 50 ml

Digestion Buffer (RNase protection)

NaCl	299 mM
LiCl	100 mM
Tris (pH 7.5)	10 mM
EDTA	5 mM

Elution Buffer

Tris. Cl (pH 7.6)	10 mM
EDTA (pH 8.0)	1 mM
SDS	0.5 %

Fixative (10% Formalin)

$NaH_2PO_4.2H_2O$	4.5 g

Na_2HPO_4	6.5 g
Formalin(40%)	100 ml
Distilled H_2O, up to	1000 ml

Formamide Loading Dye (RNase protection)

Formamide	50 ml
EDTA (0.5 M)	1 ml
Xylene cyanole	50 mg
Bromophenol blue	50 mg

Glucose Buffer

Gluose	50 mM
Tris-HCl (pH 8.0)	25 mM
EDTA	10 mM

Hybridisation Buffer (10X) [RNase protection]

NaCl	4 M
EDTA	10 mM
Pipes (piperazine-N,N' bis [2-ethanesulfonic acid])	0.4 M

LB (Luria-Bertani) Medium

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Added water to 950 ml and adjusted the pH to 7.4. Made up to 1 l with water and autoclaved at 15 p.s.i for 20 min.

MOPS Buffer (10X)

EDTA	10 mM
Sodium acetate	50 mM
MOPS	200 mM
pH	7.5

NP-40 Lysis Buffer

NP-40	0.5 %
KCl	90 mM
Magnesium acetate	1 mM
Hepes (pH 7.6)	10 mM
2-mercoptoethanol	2 mM

PBS (Mg^{2+} and Ca^{2+}-free)

NaCl		8 g
Na_2HPO_4		1.45 g
KCl		0.2 g
KH_2PO_4		0.2 g
H_2O	to	1 l

Polyacrylamide (6%)/Urea (7 M) Gel Mixture

Acrylamide/urea solution	15 ml
TEMED	15 µl
Ammonium persulfate (50%)	15 µl

Potassium Acetate Solution

Potassium acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml
pH	4.8

Prehybridisation Solution

Dextran sulfate	1.6 g
Formamide	6.4 ml
SSC (20X)	3.2 ml
Tris (2 M, pH 7.4)	16 µl
Denhart's solution (100x)	160 µl
ssDNA (10 µg/ml)	40 µl

Proteinase Digestion Buffer

Tris.Cl (pH 8.0)	0.2 M
EDTA (pH 8.0)	25 mM
NaCl	0.3 M
SDS	2 %

RNA Dye Mixture

Formamide	720 µl
MOPS buffer (10X)	160 µl
Formaldehyde	260 µl
H_2O	180 µl
Glycerol (100%)	10 µl
Bromophenol blue	5 %

RNA Extraction Buffer

NaCl	0.14 M
$MgCl_2$	1.5 mM
Tris. Cl (pH 8.6)	10 mM
Nonidet P-40	0.5 %
Dithiothreitol	1 mM

SSC Buffer (20X)

NaCl	3 M
Sodium citrate	0.3 M
pH	7.0

Succinate Buffer (0.1 M)

Succinic acid	11.8 g
Sodium azide	100 mg
Water, up to	1000 ml
pH	4.15

TBE Buffer (10X)

Tris. Cl (pH 7.4)	0.5 M

Boric acid	0.5 M
EDTA (pH 8.0)	20 mM

TE Buffer

Tris. Cl (pH 7.4)	10 mM
EDTA (pH 8.0)	1 mM
pH	7.4