EXHIBIT M

Med. Oncol. & Tumor Pharmacother. Vol. 3, No. 3/4, pp. 159–164, 1986
Printed in Great Britain

0736–0118/86 $3.00 + .00
Pergamon Journals Ltd.

PHYSIOLOGIC AND MOLECULAR BIOLOGY OF ERYTHROPOIETIN

A. J. ERSLEV and J. CARO
Cardeza Foundation for Hematologic Research, Thomas Jefferson University, Philadelphia, PA, U.S.A.

(*Accepted* 29 *May* 1985)

The gene for erythropoietin, the first growth and differentiation factor to be identified, has now been cloned and a recombinant erythropoietin is ready for clinical trials. This molecular achievement has also led to the identification of its *m*RNA in liver and especially in kidney tissue and here in the extra glomerular fraction. A radioimmune assay has been developed and its shows, as anticipated, low levels of erythropoietin in patients with kidney disease. However, in all other anemias, including the anemias of cancer, the levels of erythropoietin do not seem to be affected by the kind of disease but only by the degree of anemic hypaxia. The action of recombinent erythropoietin appears to be directed at surface receptors which increase in density on progenitor cells as they mature from early BFU-E to late CFU-E. These findings have led to an updating but not to a radical change in our concept of the feedback circuit which controls red cell production and the size of the red cell mass.

Key words: Erythropoietin.

INTRODUCTION

Erythropoietin, a regulatory growth and differentiation factor for erythroid progenitor cells, has recently been fully sequenced, and its gene isolated, cloned[1–3] and located to chromosome numbers 6[4] or 7.[5] These molecular achievements have resulted in recombinant synthesis of the pure hormone with about 1 μg in 1985 having the same biologic activity as 50 ml erythropoietin containing plasma in 1953[6] (Fig. 1).

At the present recombinant erythropoietin is undergoing phase I clinical trials in patients with anemia of chronic renal disease and, if as effective as anticipated, should be on the market in a few years. In the meantime, however, the sequenced human and murine erythropoietin genes and their products promise to elucidate the biologic feedback circuit which maintains the red cell mass at a size optimal for oxygen transport.

Much is known about this feedback circuit and it seems almost certain that its basic structure will remain valid (Fig. 2). However, how and exactly where the tissue oxygen tension is translated into erythropoietin production and how erythropoietin triggers proliferation and differentiation of erythroid progenitor cells are still unanswered questions.

Address reprint requests to: Dr A. J. Erslev, Cardeza Foundation, 1015 Walnut Street, Philadelphia, PA 19107, U.S.A.

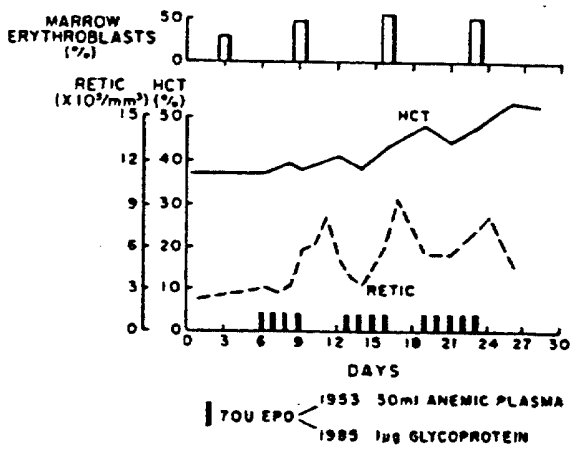

Fig. 1. The reticulocyte, Hct and marrow erythroblast response in normal rabbits in 1953 to 50 ml of plasma obtained from anemic rabbits and given four to five times a week for three weeks. The same response could be achieved now by giving 1 μg of pure recombinant erythropoietin a day for the same period of time.

RENAL BIOGENESIS

Since the classic study in anephric rats by Jacobson and co-workers in 1957[7] it has been generally accepted that the kidney is the main site of erythropoietin production. Recent determinations of erythropoietin in renal extracts have confirmed this

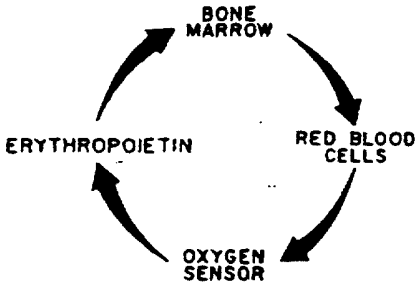

Fig. 2. The feedback control which maintains the red cell count at a number optimal for the delivery of oxygen to an oxygen sensor.

conclusion and shown that it appears in kidney tissue within 30 min from the onset of a hypoxic stimulus and before it can be demonstrated in plasma.[8–10] It reaches a maximum 6–10 h late and then decreases gradually in parallel with its concentrations in plasma (Fig. 3). DNA and RNA probes for erythropoietin *m*RNA have shown that the presence of erythropoietin in the kidneys is not due to excretion but due to actual synthesis in this organ.[11,12] The evolutionary choice of the kidney as the site of oxygen sensing and erythropoietin synthesis has been somewhat puzzling but actually makes a great deal of sense. In contradistinction to most organs, oxygen consumption in the kidney depends on blood flow since oxygen is primarily used for sodium reabsorbtion of the glomerular filtrate.[13] Consequently, in anemias in which plasma flow is increased oxygen consumption is also increased and the kidneys will become hypoxic and active in erythropoietin synthesis (Fig. 4). In polycythemia, on the other hand, the blood flow is decreased because of increased viscosity but because of the decreased filtration rate and oxygen consumption, the kidney will not become hypoxic or release erythropoietin.[14] This fortunate balance between oxygen consumption and blood flow prevents the creation of a vicious circle in which sluggish blood flow in severe polycythemia would cause hypoxia, erythropoietin production and more polycythemia.

The exact cellular source for erythropoietin production in the kidney is still unknown. Direct assays of crude tubular and glomerular preparations have suggested the tubules as the source,[15] a conclusion supported by the fact that tubular-derived cysts and tumors occasionally contain and produce erythropoietin. On the other hand, the mesangial cells of the glomeruli have been cultured and shown to produce small amounts of erythropoietin *in vitro*[16] and the tubular versus glomerular origin of erythropoietin is far from settled. An

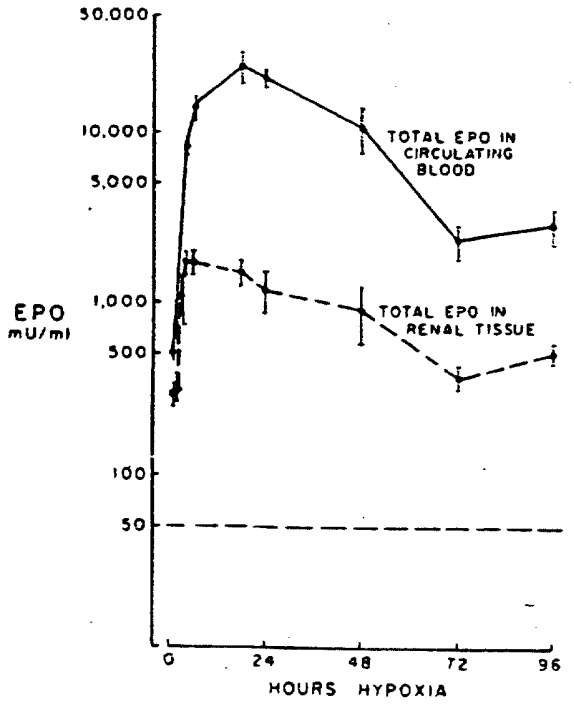

Fig. 3. Rats exposed to 0.4 atm oxygen in a pressure chamber will respond with a rapid rise in the erythropoietin content of their kidneys and in circulating blood. The erythropoietin in this study was measured by bioassay in hypertransfused mice.

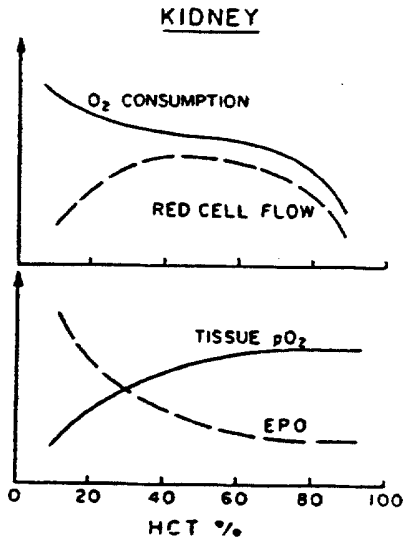

Fig. 4. The relation in the kidney at various hematocrits between oxygen consumption due to sodium reabsorbtion of oxygen supply due to red cell flow as well as the consequent renal tissue oxygen tension and erythropoietin production.

attractive compromise would be that erythropoietin is produced by the juxtaglomerular apparatus. Actually, renin and erythropoietin are closely related functionally and it has been proposed that they are derived from a common precursor substance.[17] However, careful matching of the genes for renin and erythropoietin have shown a complete lack of homology, which rules out such relationships.[1] The identification of *m*RNA for erythropoietin in cellular suspension using *c*DNA or reverse *m*RNA probes may not resolve this issue since it is difficult to accomplish a clean physical separation of glomeruli from tubules and the use of *in situ* probes will probably be needed in order to provide a definite answer.

HEPATIC BIOGENESIS

Clinical and experimental observations on anephric humans and animals have made it clear that the kidney is not the sole source of erythropoietin. About 10–15% is made extrarenally with most, if not all, being produced by the liver.[18] Hepatic production is, like renal production, inversely related to oxygen supply, but the exact intrahepatic source is unknown. Since it has been claimed that macrophages throughout the body are capable of synthesising erythropoietin,[19] the Kupffer cells are favored by some,[20] but other workers favor hepatocytes primarily because some hepatomas will synthesise erythropoietin both *in vivo* and *in vitro*.[21] Careful studies of fetal lambs and rats have shown that erythropoietin during fetal life is synthesised by the liver with a liver–kidney switchover within the first weeks of neonatal life.[22,23] Extracts from such erythropoietin-producing fetal livers have been used to identify erythropoietin *m*RNA and eventually to clone the erythropoietin gene.[1]

RATE OF PRODUCTION: BIOASSAY VERSUS RADIOIMMUNE ASSAY

The uncertainty in establishing the exact cellular source of erythropoietin has added to the difficulties in explaining some of the observations made on the level of erythropoietin found in circulating blood. These levels appear to depend almost exclusively on the rate of production since the volume of distribution is determined by the circulating plasma volume, and the rate of destruction and excretion seems to depend on plasma concentration alone.[24] Bioassays of whole plasma and plasma extracts have provided data on the relationship of bioreactive erythropoietin in plasma to hematorcrit in individuals with anemias.[25] The relationship in patients with anemias not complicated by chronic disease or renal failure suggests that a reduction in hematocrit from about 45% to 20% will result in a three-log increase in erythropoietin titers but only a 5–10 times increase in the rate of red cell production (Fig. 5). This large demand for erythropoietin by erythroid tissue may be caused by the fact that erythropoietin is needed not only for erythroid progenitor differentiation to erythroblasts but also for recruitment of CFU-E from earlier and possibly more erythropoietin demanding BFU-E[26] (Fig. 6). Bioassays of plasma extracts obtained from patients with primary and secondary polycythemia have disclosed the anticipated difference and established this assay as a useful differential diagnostic tool[27] (Fig. 7). The recent development of radioimmune assays have made erythropoietin determinations on small plasma samples possible and convenient. Large numbers of samples can now be processed but the results have so far not disclosed any significant differences between the titers of bioreactive and immunoreactive

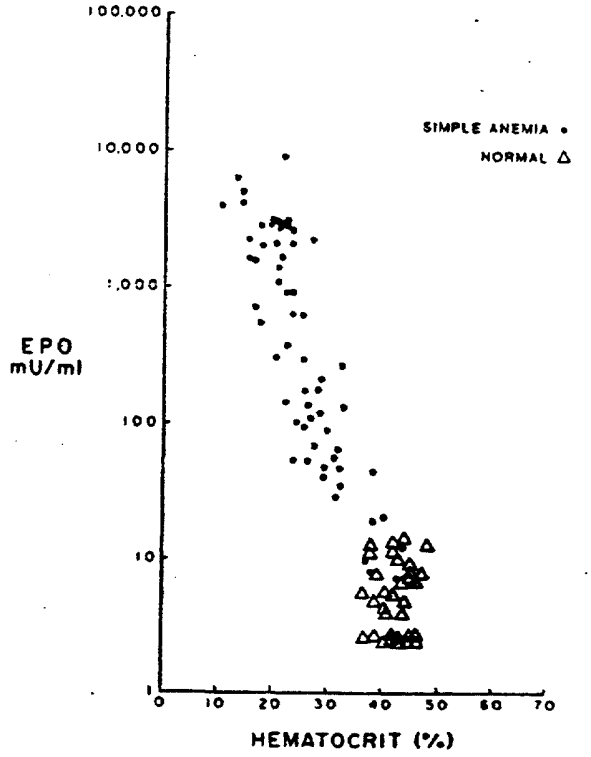

Fig. 5. Erythropoietin titers in milliunits per ml of plasma in normals and in patients with simple anemias not complicated by renal or chronic disease. The titers were all determined by bioassay in hypertransfused mice.

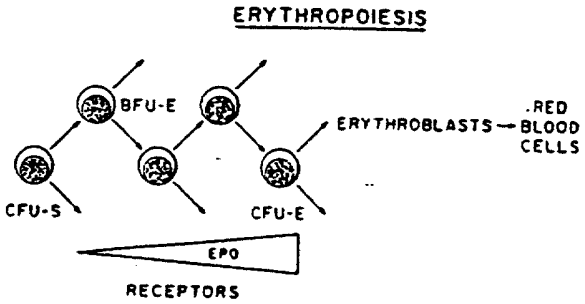

Fig. 6. The kinetics of erythroid progenitors and their assumed dependence of 'receptors' for erythropoietin. This hormone appears to act as a growth factor for progenitor cells and a differentiation factor for the transformation of CFU-E to erythroblasts.

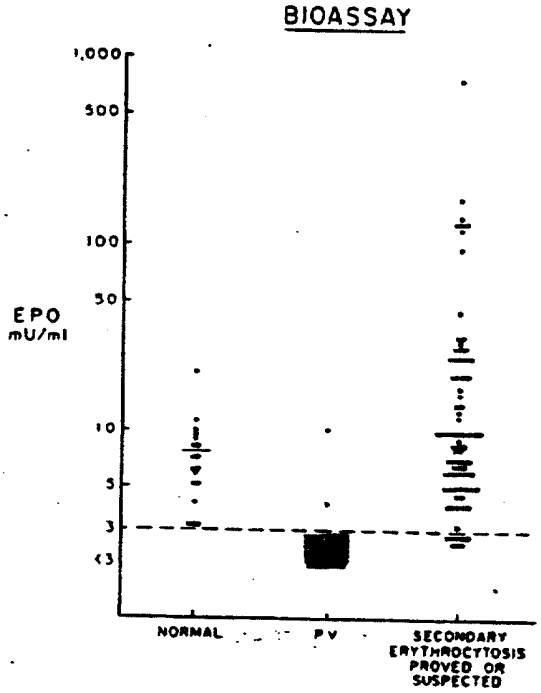

Fig. 7 Erythropoietin titers measured by bioassay in milliunits per ml in patients with established polycythemia vera (P.V.) and in patients suspected of having secondary erythrocytosis. It appears likely that patients with titers less than the limit of measurement (3 mU ml^{-1}) in the group suspected of having secondary erythrocytosis actually have polycythemia vera.

erythropoietin in plasma (Fig. 8). In the anemia of renal disease the RIA have confirmed data provided by bioassays and shown that this anemia is caused primarily by decreased erythropoietin production (Fig. 9). However, the erythropoietin titers,

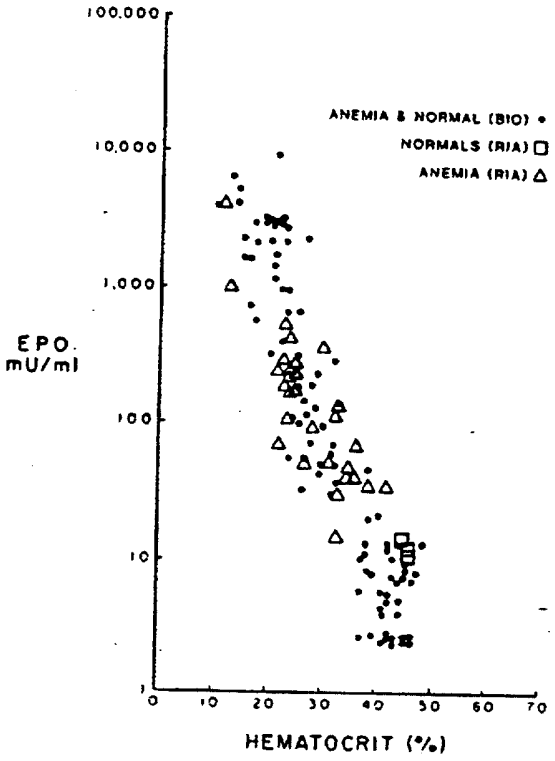

Fig. 8. Erythropoietin titers in milliunits per ml of plasma in normals and in patients with simple anemia not complicated by renal or chronic disease. It appears that the titers determined by radioimmune assay (RIA) overlap the titers determined by bioassay (BIO).

although low, are still above normal, suggesting the additional presence of an inhibitor of erythroid tissue. From a practical point of view the presence or absence of an inhibitor is not going to play a greater role in the ongoing clinical trials of recombinant erythropoietin as long as unlimited quantities of this hormone can be produced.

Studies of the concentration of bioreactive erythropoietin in patients with rheumatoid arthritis, anemia of chronic disease and sickle cell anemia have suggested that these patients have lower erythropoietin titers as compared to patients with simple anemia at the same hematocrit.[25-28] These studies have been difficult to evaluate because of wide differences among data presented and because so many other factors than hemoglobin concentration play a role in the oxygen transport of these patients. Recent studies in our laboratory using an RIA have not disclosed any significant differences between titers in these patients and titers in patients with simple anemias at the same hematocrit. Conversely, they have not shown any undue increase

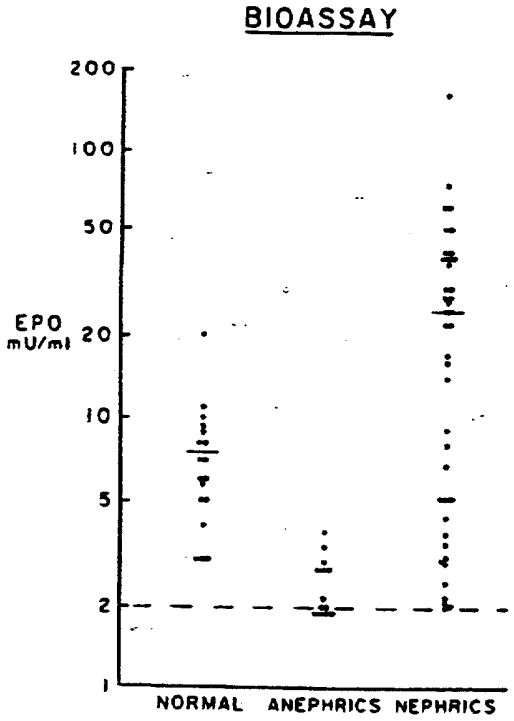

Fig. 9. Erythropoietin titers measured by bioassay in milliunits per ml in anemic renal patients with or without remnant kidneys.

in erythropoietin titers in patients with aplastic anemia or anemia due to hematologic malignancies.

RECEPTORS AND FEEDBACK

The final link in the feedback circle controlling the size of the red cell mass is the action of erythropoietin on the erythroid tissue.[29] There is little evidence for a direct action on multipotential stem cells and early committed erythroid progenitor, the BFU-E, are only marginally affected.[26] However, as mentioned above, during the BFU-E maturation towards the CFU-E the responsiveness to erythropoietin increases presumably because of the appearance of surface receptors. Erythropoietin appears to act as other protein hormones on surface receptors[30] but whether or not it is internalised or depends on secondary messengers[31] has not been resolved. Recent reports suggest that the receptor density is quite low, around 1000 per cell,[31] but these studies are based on data from iodine-labelled erythropoietin and it is not certain whether this labelling affects biologic activity. When the progenitor cell has reached the CFU-E stage, with or without a proliferative push by erythropoietin the subsequent blast transformation to erythroblast is at least critically dependent on the presence of erythropoietin.[26] The biochemical trigger in the

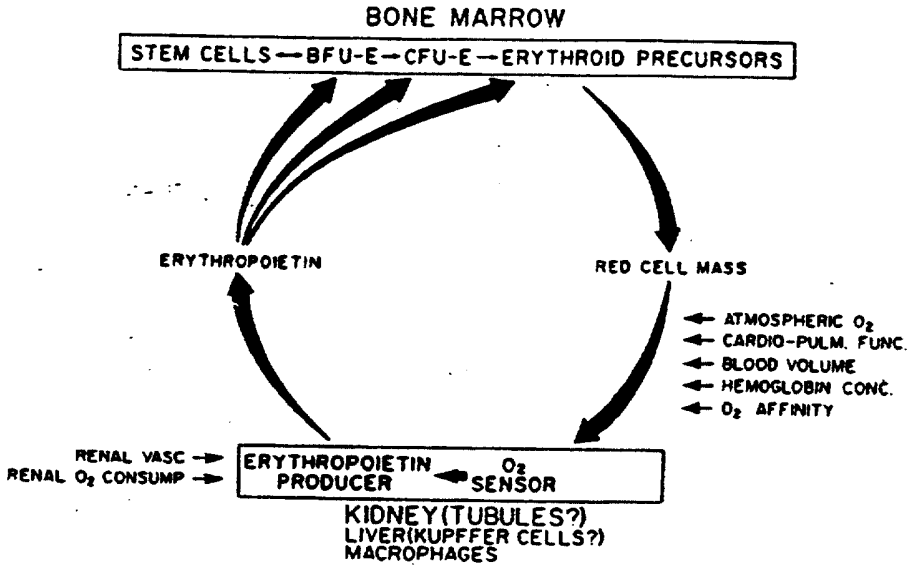

Fig. 10. An upgraded version of the feedback control depicted in Fig. 2 and explained in the text.

progenitor cell for this blast transformation is unknown but it appears to involve a process which is erythropoietin-dependent during the initial maturation of the erythroid precursor. The final maturation and release of erythroid cells have also been thought by some to be affected by the presence of erythropoietin but the evidence for this is not convincing. With the above listed findings and considerations it appears reasonable to present Fig. 10 as a 1986 model of the feedback circuit which regulates normal red cell production.

Acknowledgement — Supported in part by NIH grant No. 04612.

REFERENCES

1. Jacobs K, Shoemaker C, Rudersdorf R *et al*: Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* **313**, 806 (1985).
2. McDonald J D, Liu F-K, Goldwasser E: Cloning, sequencing and evolutionary analysis of the mouse erythropoietin gene. *Mol Cell Biol* **6**, 842 (1986).
3. Shoemaker C B, Mitsock L D: Murine erythropoietin gene: cloning, expression, and human gene homology. *Mol Cell Biol* **6**, 849 (1986).
4. LeBeau M M, Diaz M O, Rowley J D *et al*: Chromosomal sublocalization of the human erythropoietin gene. *Blood* **66**, Suppl. 1, 155a (1985).
5. Powell J S, Segal G M, Berkner K L *et al*: Chromosome localization of the gene for human erythropoietin and high level expression of clonal genomic sequences in permanent mammalian cell lines. *Blood* **66**, Suppl. 1, 159a (1985).
6. Erslev A J: Humoral regulation of red cell production. *Blood* **8**, 349 (1953).
7. Jacobson L O, Goldwasser E, Fried W, Plzak L: Role of the kidney in erythropoiesis. *Nature* **179**, 633 (1957).
8. Fried W, Barone-Varelas J, Berman M: Detection of high erythropoietin titers in renal extracts of hypoxic rats. *J Lab Clin Med* **97**, 82 (1981).
9. Jelkman W, Bauer C: Demonstration of high levels of erythropoietin titers in rat kidneys following hypoxic hypoxia. *Pflügers Arch* **392**, 34 (1981).
10. Erslev A J, Caro J, Birgegård G *et al*: The biogenesis of erythropoietin. *Exp Hemat* **8**, Suppl 8, 1 (1980).
11. Schuster S J, Shoemaker C, Wilson J, Erslev A J, Caro J: Detection of renal and hepatic erythropoietin messenger RNA in mice using an asymmetric RNA probe (abstract). *Exp Hemat* (in press).
12. Koury M J, Bondurant M C: Anemia induces accumulation of erythropoietin messenger RNA in the kidney. *Clin Res* **34**, 659a (1986).
13. Kill F: Blood flow and oxygen utilization by the kidney, in Fisher J (ed): *Kidney Hormones*, pp. 1–30. New York, Academic Press (1971).
14. Erslev A J, Caro J: Why the kidney? *Nephron* **41**, 213 (1985).
15. Caro J, Erslev A J: Biologic and immunologic erythropoietin in extracts from hypoxic whole rat kidneys and in their glomerular and tubular fractions. *J Lab Clin Med* **103**, 922 (1984).
16. Kurtz A, Jelkman W, Sinowatz F, Bauer C: Renal mesangial cell cultures as a model for study of erythropoietin production. *Proc Natl Acad Sci, USA* **80**, 4008 (1983).
17. Fyrquist F, Rosenlof K, Gronhagen-Riska C *et al*: Is renin substrate an erythropoietin precursor? *Nature* **308**, 649 (1984).
18. Fried W: The liver as a source of extrarenal erythropoietin production. *Blood* **40**, 671 (1972).
19. Rich J N, Heit W, Kubanek B: Extrarenal erythropoietin production by macrophages. *Blood* **60**, 1007 (1982).
20. Paul P, Rothman S A, McMahon J T, Gordon A S: Erythropoietin secretion by isolated rat Kupffer cells. *Exp Hemat* **12**, 825 (1984).
21. Nielsen O J, Schuster S J, Caro J, Erslev A J: Erythropoietin production in a human hepatobastoma cell line. *Blood* **66**, Suppl. 1, 158a (1985).
22. Zanjani E, Ascensao J L, McGlave P B *et al*: Studies on the liver to kidney switch of erythropoietin production. *J Clin Invest* **67**, 1183 (1981).
23. Lucarelli G, Porcellini A, Carnevali C, Carmena A, Stohlman, Jr, F: Fetal and neonatal erythropoiesis. *Ann NY Acad Sci* **149**, 544 (1968).
24. Steinberg S E, Garcia J F, Matzke G R, Mladenovic J: Erythropoietin kinetics in rats: generation and clearance. *Blood* **67**, 646 (1986).
25. Erslev A J, Caro J, Miller O, Silver R: Plasma erythropoietin in health and disease. *Ann Clin Lab Sci* **10**, 250 (1980).
26. Iscove N N: Erythropoietin independent stimulation of early erythropoiesis in adult marrow cultures by conditioned media from lectin stimulated mouse spleen cells, in Golde D W, Cline M J, Metcalf D, Fox C F (ed): *ICN–UCLA Symp on Hemopoietic Cell Differentiation*, pp. 37–82. New York, Academic Press (1978).
27. Erslev A J, Caro J: Pathophysiology and classification of polycythemia. *Scand J Haemat* **31**, 287 (1983).
28. Sherwood J B, Goldwasser E, Chilcote R, Carmichael L D, Nagel R L: Sickle cell anemia patients have low erythropoietin levels for their degree of anemia. *Blood* **67**, 46 (1986).
29. Spivak J L: The mechanism of action of erythropoietin. *Int J Cell Cloning* **4**, 139 (1986).
30. Roodman G D, Spivak J L, Zanjani E D: Stimulation of erythroid colony formation *in vitro* by erythropoietin immobilized on agarose-bound lectins. *J Lab Clin Med* **98**, 684 (1981).
31. Sawyer S T, Krantz S B, Goldwasser E: Receptor-mediated endocytosis, and degradation of erythropoietin in Friend virus-infected erythroid cells. *Blood* **66**, Suppl. 1, 161a (1985).