EXHIBIT 3
Part 4 of 5

the "inactive" tyrosines is iodinated instead. A preparation of ^{125}I-epo with 75% of its original activity would permit facile binding studies as well as studies of internalization.

The alternative (not mutually exclusive) approach will be to modify the sugars. We know that asialoepo is active, in culture. We plan to use ^{32}P to phosphorylate asialoepo chemically, by a carbodiimide reaction.[34] Since we will have removed the negative charges with the removal of sialic acid by neuraminidase, addition of a few negative phosphoryl charges should not materially affect the activity. It will be important, nevertheless, to determine whether the binding constant is the same as that found for ^{3}H-epo. It would also be important to determine whether in vivo activity was regenerated.

A third approach will be to use IW32 cells actively synthesizing epo, and add enough ^{35}S methionine and cysteine to get very high specific activity, this will entail purification of mouse epo, but since we know that our monoclonal antibody recognizes mouse epo, it should be possible with the help of an immunoaffinity column, followed by a wheat germ agglutinin column and if needed, hydroxylapatite chromatography to obtain small amounts of homogeneous epo from the culture medium. There will be no difficulty in establishing homogeneity by SDS gel electrophoresis since the silver stain method[35] can detect a few ng of epo and, fluorography of the labeled material by a micro slab gel electrophoresis can confirm the results with an even smaller amount.

With adequately labeled, active epo we plan to reinvestigate the metabolism of epo in vivo.[24] Our most recent studies were done with iodinated epo devoid of activity and it is possible that it was handled differently from active epo. We will repeat the experiments, both with respect to clearance rate, appearance in urine and tissue content. If we are successful in finding a smaller, active epo preparation (see below) we will do similar experiments with it as well.

Binding of epo

We plan to determine, in populations of normal mouse and rat bone marrow, both the fraction of cells, that can bind fluorescent epo and the amount of labeled (^{3}H,^{125}I or ^{32}P if possible) epo bound at saturation. While we can use the methods already developed for this measurement of frequency, we plan to simplify it by use of Covaspheres (fluorescent beads) covalently linked to the $F(ab)_2$ fragment of monoclonal anti-epo. These, in principle should react with epo previously bound to cells with epo receptors and permit their enumeration. These two determinations will permit expression of the specific binding on a per cell basis. The usual "binding as a function of ligand concentration" type of measurement could then be made and, if there is a straight-line Scatchard plot, (i.e., a single class of receptors), will permit the estimation of both K_D and receptor number(n). This method will provide an approximate answer to the question of what quantitative changes (if any) in K_D and n occur as cells progress along the differentiation pathway. We will start with marrow from artifically polycythemic mice which are devoid of CFU-E and contain 8 day BFU-E. The progression can be followed as a function of time after such mice are given a dose of epo. We would then make the same measurements on a normal mouse marrow population which, with respect to epo binding, contains CFU-E and later cells predominantly. It would probably not be worthwhile to use cells from anemic mice for the later stages since they would come from animals with a high titer of endogeneous epo and may have their cellular receptors saturated, as appears to be the case for purified CFU-E.[36]

If these experiments are successful they will permit the extension of the method to the study of human disease states. We plan, in collaboration with Dr. Sanford B. Krantz of Vanderbilt University, (see letter in appendix) to measure n and K_D for normal marrow and marrow from patients with polycythemia vera in order to determine whether the magnified sensitivity to epo in these disease[37] is a consequence of a change in K_D or n or both, i.e., whether this disease is a receptor "mutation," as appears possible. Other anemias in which there is an erythropoietic response inappropriate with respect to the measured epo titer will also be studied with the hypothesis that

the anemia may be due to receptor deficiency.

We will also be engaged in the purification and characterization of the FVA cell epo receptor. The immobilized epo column, already referred to, will be used to isolate the solubilized cell membrane receptor. We have already shown that membrane preparations from FVA cells can bind epo specifically, with essentially the same characteristics as whole cells. We have also shown that bound epo can be cross-linked to the receptor with disuccinimidyl suberate and that an epo:receptor sub-unit may be detected on a denaturing gel. Our intent is to further purify this binding sub-unit, and if possible, the other receptor sub-units. We will then investigate such standard phenomena as tyrosine phosphorylation of a receptor sub-unit as a result of epo binding and whether there is a subunit with tyrosine kinase activity.[38] We plan to purify the binding subunit to homogeneity, determine partial, or complete, amino acid sequences and construct an oligonucleotide probe based on a unique region of the receptor, this probe will then be used to screen a mouse cDNA library (already prepared) for the receptor gene as a start in the molecular cloning of that gene. When the gene is cloned the complete sequence will be determined.

One hypothesis that will be tested with the cloned receptor cDNA is as follows: erythroid differentiation may depend only on the target cell having a specific epo receptor, i.e., the presence of the active receptor on the cell surface is sufficient for all the subsequent events in epo action. We plan to test this hypothesis by transfecting non-erythroid cells, e.g., 3T3, with the receptor gene linked to the thymidine kinase gene. Selection of thymidine kinase positive cells in HAT medium should result in receptor positive cells as well. This can be tested by either Southern blot analysis[39] using the probe mentioned above, or if the gene is expressed, by demonstrating that epo is bound to the 3T3 cells; it should not be bound to cells not transfected, nor to cells transfected only with the thymidine kinase gene. If receptor expression occurs, i.e., epo is bound specifically (competition with unlabeled epo) we can then determine whether interaction with epo results in globin genes being expressed. We will use the Northern blot analysis[40] using cloned mouse globin genes now available and used in another context in this lab.

Another important reason to study the structure of the epo receptor comes from the recently discovered homology between the epidermal growth factor receptor and the oncogene V-erb-B.[41]

Cloning of epo

Since the human[3,4] and monkey[4] epo genes have been cloned and the former expressed in eukaryotic cells, further cloning efforts will not be difficult, despite the fact that, to extrapolate from the monkey, there is only a single copy of the gene. We have started, and will continue, to clone both the normal mouse and IW32 epo genes in order to understand their comparative structures and the relationship between the murine erythroleukemia virus and the expression of the epo gene. The cloning strategy for the two genes follows: we will use the monkey epo cDNA probe to select a sub set of IW32 (or anemic mouse) DNA samples for cloning in the vector λL47. The enriched library will be packaged and amplified, after screening for the appropriate clones with the same probe. DNA sequencing will be done in the M13 system [42] by the Sanger method. [43]

Once we have succeeded in this endeavor, it will not be difficult to clone epo genes from other species, in order to seek conserved sequences that can yield data on important structural features, using the same strategy and, in fact, the same probe.

Although, as indicated in another section of this proposal, we plan to study synthetic peptides with binding activity toward epo receptors, it is possible that the other aspect, that of seeking a smaller peptide with epo activity on target cells, may not be successful. If it is not, the synthetic approach to amino acid replacement as a means to determine structure-function relationships would not be feasible. Since it is a potentially important aspect of epo biochemistry, we plan to also use the alternative approach. With a cloned epo gene, especially a cDNA clone which can

be used to form active epo in transfected eukaryotic cells,[4] it will be possible to modify the gene, e.g., by synthesis of oligonucleotides with alternative codons to replace amino acids in the active region (site specific mutagenesis). In general, this method involves the synthesis of the small part of the gene in question, using alternative codons, followed by linkage to restriction enzyme fragments of the original gene to reconstitute a modified epo gene. For example, we have information that tyrosine, trytophan and lysine residues may be intimately involved in binding and biological activity. We could engineer a new epo DNA with either similar residue replacements (phenylalanine for tyrosine and arginine for lysine) to determine the effect on activity. Similarly the effects of substitution of dissimilar amino acids would be studied. It is possible, by this type of approach, with all of the residues in the connecting region, that we could develop both a more active epo-like material and/or a specific inhibitor of epo action. We recognize that these proposed experimental approaches are new departures for this lab but, we are confident that the techniques are accessible (there is an oligonucleotide synthesizer in the department at present) and that the possible results are worth the effort.

We plan, in addition, to use the epo cDNA probe in collaboration with Dr. Janet Rowley (see letter in appendix) to localize the human epo gene to its chromosome by in situ hybridization.

Internalization

In view of our findings on the relatively small number of receptors per FVA cell,[6] the questions of whether epo is internalized and, if so, what role internalization may play in the induction of erythroid differentiation becomes harder to answer. Because, inherently, an isotope with a long half-life, such as ^{3}H, cannot provide an epo preparation of high enough specific activity for studies of possible internalization of a very few molecules of epo, we will have to use ^{125}I, ^{32}P or ^{35}S labeled epo as suggested earlier. When feasible, epo labeled with one or more of these isotopes to high enough specific activity will be used to determine whether any epo becomes intracellular, by direct analysis of washed cells.

Another, less direct, approach will be to permit FVA cells to bind labeled epo over a long period (18-24 hours) and remove external epo by washing and mild trypsinization. The cells will then be washed free of the trypsin, treated with trypsin inhibitor and incubated with and without unlabeled epo. We will look for externalized label, whether of native size or degraded. Finding degraded epo in the medium will mean that it had been internalized. A control experiment with cytochalasin B, chloroquine and/or monensin which inhibit lysosomal breakdown of endocytosed ligands will also be done. These types of experiment will have to be done with large cultures, too large to be able to determine radioactivity in the total cells, but the large volume of medium can be concentrated by lyophilization before examination on HPLC or Western blots for size of labeled, immunoreactive material.

Fragments

Our data strongly suggest that the active site, probably the site that binds to receptors, is in the connecting region between the two protease resistant domains.[11] Since partial proteolysis destroys the region of epo we wish to study, we plan to use other techniques to prepare and isolate a peptide containing the active sites; among these techniques is hydrolysis with CNBr. Our preliminary data suggest that epo contains 2 methionine residues so that there should be three peptides formed. One recent experiment shows that after CNBr cleavage there are formed components of size, 2K,3K,11K,14K and 28K by SDS gel electrophoresis. Some of these must represent products of incomplete cleavage. We propose to isolate these peptides by reverse phase HPLC and determine which, if any bind to epo receptors on FVA cells. We can measure binding directly if the peptide can be iodinated or tritiated or indirectly by competition for native epo. In either case we will also test for inhibition of epo activity in the marrow culture system working under the assumption that binding of a fragment would be non-productive biologically. Of course, we will test the biological activity of these fragments as well by the same culture method, since a small,

active, epo-like peptide would be of great interest.

If we find a CNBr fragment with either biological activity or inhibitory activity we will then accumulate enough to determine its sequence by the gas phase micro sequencer, in order to understand the structure of the binding region of epo. This understanding would also make it possible to synthesize either an active epo-like peptide or a specific inhibitor of epo action.

In addition to CNBr we plan to use endoproteinase arg C, which does not cause cleavage of the connecting region. By first denaturing epo, to expose the other arginine residues and alkylation of the cysteines we should get significant cleavage at non-active sites. The peptides so obtained will be isolated and tested for activity, binding and inhibition of biological activity.

Biogenesis of epo

The isolation, culturing and cloning of a mouse cell line (IW32)[5] that secretes substantial amounts of epo into the medium has made it possible to now study some aspects of the biogenesis of epo. IW32 cells are already being grown in this laboratory and we have shown that labeled epo can be produced in culture. We plan to use both ^{35}S-methionine and/or ^{3}H-leucine plus either ^{3}H on ^{14}C glucosamine or fucose to trace the pathway of biosynthesis. If, as found for most secretory proteins, epo is synthesized as a large precursor protein which is then translocated and glycosylated before being shortened to the secreted size, we should be able, by a combination of biolabeling and sizing on HPLC columns, to determine the events in epo synthesis and release. In order to be reasonably sure that the products we follow are related to epo we will routinely test them for reaction with anti-epo. We have developed a simple and rapid binding assay, based on the use of HPLC sizing columns, that permits us to determine whether epo or anti-epo is in a complex significantly larger in size than the starting material.[12] Another method being set up in this lab, is the "Western" blot technique[27] that permits the determination of approximate size of immunoreactive material.

The evidence presented in the Progress Report on extraction of beef kidney epo strongly suggests that epo is held in a membranous complex in the tissue. The findings on rat kidney after Ni_3S_2 injection suggest that there may well be a biologically inactive, immunoreactive precursor.[23] We plan to use these findings as the start of the study of intracellular epo in IW32 cells. We will look for proteins and glycoproteins of different size than secreted epo after opening up cells which had been grown in the presence of labeled amino acid and of labeled glucosamine. Cell breakage will be done with either sonication, detergent or a micro decompression bomb[17].

If epo is synthesized by the conventional schema, we expect that the first immunoreactive component formed should be the membrane bound epo precursor protein (molecular weight greater than 22K). There should then follow, with time, glycosylated forms of a range of molecular sizes, but without sialic acid. These forms should contain glucose. As the high mannose oligosaccharides are trimmed (loss of glucose and mannose) to smaller forms which then are further glycosylated to the final size (molecular weight approximately 34K); native epo should appear last, and be found in isolatable secretory granules.

By use of inhibitors of glucose trimming such as nojirimycin (5 amino 5 deoxy-glucopyramose;[44] it should be possible to halt epo formation at the stage of high mannose, glucosylated oligosaccharides. This might also inhibit secretion which could be determined as outlined below. Another inhibitor, swainsonine,[45] could block conversion of "trimmed" oligosaccharides to the complex type resulting in a smaller, less highly charged glycoprotein. The more traditional inhibitor, tunicamycin[46] which blocks the initial steps of glycosylation by blocking synthesis of dolichol pyrophosphate oligosaccharides, could be used to verify the formation of intracellular epo protein independent of glycosylation.

Regulation of biogenesis

In addition to the path of glycoprotein biosynthesis, we are planning to study the mechanism by which cells regulate epo synthesis or epo gene expression. Animals

in vivo, respond to hypoxia by increased de novo epo secretion; the mechanism by which reduced oxygen triggers this response has never been understood. We plan to first determine whether the amount of epo secreted by IW32 cells can be increased by the sorts of stimuli that act in vivo; these are hypoxia, testosterone,[47] cobalt salts,[48] cyclic AMP[49] and prostaglandins.[50] If one or more of these has an effect on IW32 we will have an admirable tool to study the regulation of epo secretion. If the constitutive secretion by IW32 cells is maximal, i.e., if we cannot increase it by "physiological" stimuli we will do the same types of experiments with fetal liver cells or with normal kidney cells from mice subjected to the various stimuli and if possible with cultured normal kidney cells exposed to the same stimuli. (The experiments outlined below will serve for both IW32 and normal cells). The use of normal cells will require the more sensitive RIA we plan to develop. It will also be possible to do this sort of experiment in whole mice subjected to the various stimuli and examining the kidney tissue along with lines suggested below. This expedient will be not used until we are convinced that neither IW32 nor isolated kidney cells will respond to appropriate stimuli in a physiological manner.

One set of tentative hypotheses involves control by hypoxia, or the other stimuli, at one (or more) of the following levels prior to secretion: the physical state of the epo genic DNA, transcription of the epo gene, processing of the epo mRNA, microsomal translation of the message, glycosylation and secretion.

The state of epo genic DNA may be determined, as in other studies of gene expression by studying the sensitivity of the specific epo gene to DNase.[51] by use of the Southern technique which, with the labeled cDNA probe now available in our lab, can detect, by size determination on electrophoretic gels, enzymic cleavage within the gene, or near it on the chromosome. There is precedent for increased DNase sensitivity accompanying new expression of some genes. Similarly the degree of methylation of a particular gene, detected by differential hydrolysis by two restriction enzymes,[52] Msp-I which will cut the sequence CpCpCpGpGp whether or not the middle C is methylated, and Hpa II which will not cut there if the C is methylated. Once again, the Southern blot technique will be used to determine change in possible methylation of the epo gene.

If erythropoietic stimuli, by some mechanism not yet postulated, act on the state of the epo gene, we will have to look for mechanisms for such an effect. Either the stimulus acts directly on the epo-secreting cell, possibly by way of a cytoplasmic mediator, or hypoxia (and/or the other stimuli) act directly on some other sensitive tissue which releases a molecular effector, the target of which is the epo secreting cell.

This latter possibility extends rather generally to all of the mechanisms we plan to study and we will devote effort to study of it. One approach will be to examine the immediate effect of hypoxia on a possible plasma-borne substance. We will collect blood from animals after an exposure to hypoxia too short to result in epo production (determined by RIA on the serum at short intervals) and test the plasma on normal kidney cells in culture to determine whether increased epo secretion results. If so we will be obliged to work on the chemical nature of this substance, by new techniques as well as methods we have been using for more than 25 years. Of course, the more obvious compounds such as prostaglandins, steroids, known peptides and other hormones will be screened before searching for any unknown factor.

We will study both the possible effects of stimuli on transcription and mRNA processing by using the epo cDNA probe. The Northern blot technique will be the method of choice. We will use the probe to determine when, in response to stimuli, epo mRNA is made and whether it may be made constitutively without any stimulus. The RNA to be run on the electrophoretic gel will be extracted by the guanidine thiocyanate method.[53] Preliminary experiments in this lab have already shown that the monkey epo cDNA probe can be used to detect the single copy epo gene in Southern blots of about 10 ug of DNA from normal mouse kidneys. This demonstrates that the sensitivity of the method is adequate for the experiments we plan. If, to our surprise, we need additional sensitivity for Northern and Southern blots, we will use the recently described method of Studencki and Wallace[54] which can improve detection by at least one order

of magnitude. Quantitation of the Northern blots will permit an interpretation as to whether each of the stimuli act on epo gene transcription, whether they all have the same mode of action, and if not, will permit sorting out which of the several stimuli should be studied by procedures that examine steps later than transcription and which should be followed up by studies of control of transcription.

Regulation of translation will be studied by measuring the amount of epo mRNA associated with polysomes and correlating that with the amount of epo polypeptide synthesized. For the former we will isolate polysomes, prepare the polyA+RNA and do Northern analyses; quantitating by scanner measurements. For the latter we will use the anti-epo column after biolabeling and addition of carrier epo protein. If regulation is at the translational level, i.e., if the rate-determination steps are post transcriptional, then inhibition of RNA synthesis should have no short-term effect on stimulated epo formation. We will study that possibility using standard inhibitors of transcription.

If the regulatory steps are post translational, e.g., on intracellular transport to the Golgi or on glycosylation, we would expect to find epo protein made in excess of glycosylated epo. That can be tested by a combination of RIA, labeling with glucosamine and separation of antigen-antibody complexes on an HPLC sizing column.

Lastly, we can surmise that fully active, native epo is stored in secretory granules and that the stimuli may act on secretion only. The early data from Schooley[55], showing that inhibition of RNA and protein synthesis caused inhibition of the epo response to hypoxia, was interpreted to mean that de novo epo synthesis was affected by the stimulus. This interpretation does not take into account the possibility that secretion of pre-formed epo may require the de novo synthesis of some other protein. We can now test that possibility by determining whether the first epo secreted is uniformly labeled with both amino acids and sugars. This will require biolabeling and isolation by an immunoaffinity method after addition of carrier, either in vitro or in vivo (or both). Secretory granules can be isolated by density gradient sedimentation and the amount and state of the epo contained in them studied as a function of time. By biolabeling we should be able to determine whether pre-existing epo in granules is replaced with newly synthesized epo or whether all epo generated in response to a stimulus is newly formed. We are confident that the recent advances in this field now permit such experimentation.

As indicated in the Progress Report, we plan to continue our study of a solid-phase RIA based on the monoclonal anti-epo. This method has, in most essentials, been worked out and it now needs modification to increase the sensitivity. We plan to do this by reducing the background bound radioactivity (anti-rat antibody bound to rat monoclonal antibody on the polystyrene bead to which epo was bound). This may be done by improved washing of the bead with high salt if the bonding is electrostatic or low salt if it is apolar. Transfer of the bead to a secondary tube would also help reduce the background. Another expedient will be to compare very highly labeled (^{125}I) second antibody with similarly labeled protein A. We anticipate this problem to be solved rather quickly.

Lastly, we will continue our collaborative studies of epo levels in experimental animals and in patients with laboratories doing research that may be of primary interest in this laboratory.