EXHIBIT 3
Part 3b of 5

exponential function. It was 3.5±0.2 hours in normal rats and 4.4±0.3 hours in rats with ligated renal pedicles. The labeled epo did not accumulate in the kidney. We have not yet determined where the labeled epo does accumulate, but because of the uncertainty about the ^{125}I-epo, prefer to re-investigate this problem with biologically active, labeled epo.

Extraction of epo from tissues The method for extraction of epo from tissue,[25] published from this laboratory some years ago, permitted the conclusion that the putative tissue of origin does contain epo. Since it was possible that epo was held in membrane complexes within the cell, we studied this further and found that additional epo could be extracted if a detergent (Triton X-100) were added to the buffer. For example, with beef kidney, in one experiment there was 40 times more epo in the soluble fraction when detergent was present, in another it was 35 times and for bovine fetal liver there was 77 times more. All of these are based on RIA. The chromatographic behavior of these extracts on DEAE cellulose suggests that the increase may be due largely to poorly glycosylated or sialated epo. From 70-90% of the RIA positive material from the detergent extraction does not bind to the exchanger, whereas fully glycosylated and sialated epo does.

Clinical test of epo In a very small clinical trial of pure human epo to determine effect on erythropoiesis in anemic patients with chronic renal disease, maintained on dialysis, we first obtained FDA approval to use pyrogen-free human epo in man. Two patients were given 520 u/injection, intravenously, twice a day for 10 days. A third was given 1000 u/injection every 2-3 days, immediately after dialysis, for 3 weeks. Two normal volunteers received 500 u in a single injection for clearance studies. We found no acute, subacute or chronic adverse reactions to the epo; all three patients continue on the dialysis program. There was no significant change in hematocrit in any patient; each patient, however showed an increase in reticulocyte count, with peaks at 9, 10 and 11 days. The first two patients had increased erythroid cells in the marrow and an increased plasma iron clearance rate. One of the first two patients showed an increase in red cell mass. These fragmentary data, need to be reinforced with more extensive and extended studies but they show that epo can have a physiological effect in this type of anemia. We plan to continue these studies, but not as a part of this proposal.

Solid phase RIA Some of our proposed experiments, e.g., those concerning naturally occurring "fragments" and biogenesis would be materially aided by a more rapid and sensitive RIA. We have begun to develop such a method, using a solid phase antigen. In brief the method involves immobilizing crude epo on polystyrene beads, mixing either standard epo preparations or unknowns with an excess of monoclonal anti-epo, and adding this mixture to the epo on the bead. After incubation, the bead which would have bound free anti-epo but not epo-antibody complexes, is washed and then reacted with ^{125}I labeled protein A or rabbit anti-rat IgG. The amount of antibody on the bead can thus be quantitated and will be inversely proportional to the amount of epo in the original solution. In our preliminary experiments we have found the proper conditions of time, temperature and pH to bind the crude antigen to the bead (24 hours, 37°, pH 8). The background can be reduced considerably by doing all of the steps in tubes coated with polyethylene glycol. With our monoclonal antibody, binding to the insolublized epo, occurs in 2 hours at 37° and rabbit anti-rat IgG binds to the beads in 1 hour at 37°. We propose to continue these studies to improve the sensitivity of the method and, by reducing volumes, decrease the time needed. To date we have been able to generate reasonable dose-response curves, but the minimum detected was about 12 mu. We need to get the sensitivity down by an order of magnitude or more. This may be achieved by use of smaller tubes (microfuge) for the reactions, by more highly labeled second antibody, and by reducing the amounts of bound antigen and monoclonal antibody used.

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Epo biogenesis We have started to work with the IW32 cells (sub clone 4.1) obtained from the laboratory of Dr. Bruno Varet, Hôpital Cochin, Paris. The cells can be grown in serum-free medium (RPMI 1640 supplemented with 0.1% albumin and an insulin, transferrin, selenite mixture). Under these conditions there is parallel cell growth and epo release up to about 120 hours at which time the epo content of the medium plateaus at 0.2 u/ml as the cell concentration continues to increase. Growth in the presence of fetal bovine serum is accompanied by increased epo secretion but our purposes are better served, at this time, by use of serum-free medium. Tunicamycin, which inhibits glycosylation, inhibits epo production (measured by the marrow cell culture method) by about 76%-86% (depending on concentration) at 65 hours. Incorporation of labeled leucine into what appears to be epo occurs in culture. We are in the process of confirming the identity of the labeled material at this time.

Colony-stimulating Factor Because of our interest in the possible competition between epo and colony-stimulating factor (CSF) for target cells, and because CSF is present in reasonable quantity in the crude urine concentrate we use for epo preparation, we purified the CSF.[26] The procedure involved six steps with an overall yield of 3.8% and a purification factor of 25,000. The final CSF has a potency of 1.9×10^8 units/AU, it has an apparent molecular weight of 46,000 and is a disulfide dimer. The momomer is not active. The colonies induced by this CSF contained only macrophages when mouse marrow was used. Neutralizing antibody against mouse L cell CSF (also a macrophage colony inducer) did not inactivate human urinary CSF, but did bind to it.

Cloning of epo In collaboration with the staff of Amgen, Inc., we have, jointly, been successful in cloning the monkey and human epo genes.[4] We used pure human epo to generate partial amino acid sequences, which were the basis for synthesizing mixed oligonucleotide probes. These probes, 128 different sequences of 20 residues each, were used to analyze RNA, obtained from kidneys of monkeys made anemic with phenylhydrazine, by Northern analysis. A monkey cDNA library was prepared and screened with the probe and several epo positive clones identified. The cloned cDNA; inserted in a SV-40 promoter expression vector showed expression of the epo gene amounting to about 3 u/ml secreted into the medium. The recombinant epo produced was active by RIA, bone marrow cell assay and in vivo. The monkey probe was also used to detect the single human epo gene in genomic blots and permitted the cloning of it as well.

We have also, in this laboratory, used the monkey cDNA probe to study mouse epo and have made a good start in cloning the normal mouse gene, and the gene from IW32 cells.

Epo distribution Lastly, although it does not represent any direct scholarly contribution, this laboratory has made possible a significant number of achievements in other laboratories by supplying epo to them. We have used the distribution program, initiated by the Blood Resources Division and operated through the laboratory of Dr. P.P. Dukes to make available, thoughout the world, both pure human epo and partially purified material. For the former we provided 245 vials each containing 0.7 µg of epo suitable for radioiodination and to be used as the tracer in RIA. The latter preparation amounted to 305,660 units, with a potency of 1100 u/mg of protein; it was designed to be used in cell culture experiments, as a working standard. We had removed virtually all of the CSF activity, burst-promoting activity and endotoxin. It was active in permitting erythroid burst growth from mouse and human marrow cells. With mouse cells it was non-inhibitory out to 10 u/ml. (see appendix)

Making both of these preparations available, we feel, has resulted in a significant increase in research on erythropoiesis; there is no doubt that we made possible the establishment of the RIA in several laboratories, and the results from these laboratories, have increased our undertanding of both experimental and clinical problems.

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EXPERIMENTAL DESIGN AND METHODS

Purification of epo

One early goal is to have a reproducible immunoaffinity method of purification. This would be very useful and time saving in the extension of our structural studies of epo to species other than man; especially for our proposed work on IW32 mouse epo which appears to be smaller than normal mouse and human epo. This difference may reside in the oligosaccharide structure which, in general, tends to distort size measurements by gel permeation and SDS gel electrophoresis. It will require appreciable amounts of pure epo derived from the tumor cells and/or normal mice to examine this question and immunoaffinity would be the method of choice if we can make it work with good yield, on a routine basis.

The procedures used to prepare an immunoaffinity column to date may have been faulty (yielding low capacity columns) possibly because of impure antibody or because of kinetic factors. We plan to produce large amounts of the monoclonal anti-epo by both cell culture in medium containing agammaglobulin serum and from ascites fluid made by injecting the hybridoma into nude mice. The IgG will be purified by ammonium sulfate precipitation and by using a goat anti-rat IgG immobilized on agarose and eluting the antibody with 2 M NaSCN or 2 M guanidine: 0.1 M glycine 0.3% polyethylene glycol 6,000 pH 4.2 (both of these eluting solutions have worked in the past). A small aliquot of this preparation, once the purity has been verified by SDS gel electrophoresis, will be lightly iodinated for use as a tracer. We will then determine the rate and extent of linkage of the anti-epo to "Affi-gel 10," an agarose derivative containing a 10 atom side chain and a succinimidyl ester. Using the best conditions we will prepare a working column of anti-epo. A micro column of anti-epo made using the same conditions of time, temperature and pH will then be used to determine the rate and extent of epo binding using ^{3}H-epo as the tracer. This column will be used to determine the conditions for maximum binding and elution of epo. We already know that passage of epo as slowly as 0.1 ml/hour through an anti-epo column does not result in maximal binding. These conditions will then be used routinely.

Because the monoclonal anti-epo recognizes only one of the numerous antigenic sites of epo, we plan to use a related immunochemical method as well. By use of an immobilized epo column (already prepared) using Reactigel 6X, an imidazole carbamate that links epo to agarose through its carboxyl groups, we will prepare polyclonal, monospecific anti-epo from antisera raised in rabbits. These antisera will be provided by laboratories to which we have already sent crude epo for immunization purposes and will be tested for non-human epo binding by the HPLC sizing column (TSK 3000) method used previously.[12] The monospecific anti-epo should prove very useful for epo localization studies, for "Western" blot analysis[27] as well as for purification. In general the same techniques will be used to purify the polyclonal antibody as described for the use of the monoclonal antibody. It is possible that the Western blot technique will not work with an antibody directed against native epo; if so we will prepare an antiserum to denatured (reduced and alkylated) epo and use it for the blot method.

One of these methods should permit us to purify non-human epo with minimal loss. We already have about 20 L of anemic rabbit plasma stockpiled for that purpose and are growing IW32 cells to accumulate enough medium to be able to characterize mouse (tumor) epo. Rat plasma, from phenylhydrazine treated animals will also be stockpiled for this purpose.

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Chemistry of epo

We expect, in collaboration with the Amgen staff,* to have finished the determination of the complete aminc acid sequence by the time this grant would be activated. This structure will then be studied for homology with other hormones and growth factors and with any of the known oncogenes, because of the finding that structure of platelet-derived growth-factor (β-chain) is closely related to that of the oncogene, C-sis.[28]

We will then go on to study the position of the two disulfide bridges. Our indirect evidence suggests that each compact domain contains one disulfide,[11] so that analysis of their structures separately, or of only one, when the complete sequence is known, should permit us to assign the disulfides in an unequivocal manner. Since we already have methods for isolating the two large, protease-resistant domains by reverse phase, HPLC,[11] this problem should be rather quickly solved. Because we find that the disulfides appear to be essential for proper conformation and, therefore, for biological activity, establishing the positions of the SS-bridges may help us understand the structural basis for interaction with receptors.

Another aspect of epo structure determination will also be fairly rapid, that is the calculation of regions of hydrophilicity and, therefore, of possible antigenicity by the Hopp and Woods[29] method. As soon as the sequence is available and verified this will be done. Determination of secondary and tertiary structure by physical methods, in general will require much more pure epo than is now available, so we do not plan any extensive studies in this direction for the next few years. We will, however, start on a modest program to determine whether epo can be crystallized, in preparation for eventual structural analysis. To date, only one report of a glycoprotein, with such a large degree of glycosylation, being crystallized has appeared.[30] We will attempt to use a similar method to crystallize the lead salt of epo using a systematic series of ethanol concentrations. This approach does not have a high probability of success, but it does not require much time, effort, or epo (what epo is used without success can be recovered and used for other chemical purposes), and if successful would eventually permit the three dimensional structure to be determined. It, therefore, seems to be worth the effort.

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*NOTE: Because it is not usual for a university laboratory to collaborate with a commercial laboratory, it is worth some comment. The active interchange of ideas, methods and some materials has made possible the fullest use of two sets of investigative capacities to accelerate research. For example, until this department recently obtained the gas-phase micro sequencer, it was impossible to determine amino acid sequences of very small amounts of peptides derived from epo. Such a facility was available at Amgen and our preparation and isolation of peptides, by partial hydrolysis of epo, was followed by the sequence determination of peptides. This in turn lead to the synthesis of a large number of oligonucleotide probes which were used for the successful cloning of the monkey and human genes. Neither lab in isolation could have completed this research in the time used by both labs in collaboration. This collaboration will greatly reduce the time needed for getting an ample supply of epo for experimental and possible use. It has already provided this laboratory with the monkey cDNA clone that is being used for screening for mouse epo clones.

With respect to the carbohydrate portion of epo we plan the following in order to determine the number of oligosaccharide chains, and to find out whether that number will agree with the glycosylation signal sequences (asn-X-$\frac{ser}{thr}$) that will emerge from the primary structure. We will desialate epo, and label the terminal sugar residues by mild periodate oxidation, followed by NaB^3H_4 reduction. Carrying these reactions to completion should result in each oligosaccharide being labeled. We will do a limited tryptic hydrolysis,[11] separate the two domains containing carbohydrate, determine the radioactivity per domain, and follow that by complete pronase digestion. This last step should result in free amino acids and oligosaccharide-asparagine chains. These oligosaccharides can be separated from each other (if they are different) by DEAE column chromatography.[31] There is now available a DEAE type of HPLC column (Synchron AX300) that will be tested for more rapid isolation of the oligosaccharides using orosomucoid as a model. The separatipn should then provide data based on radioactivity, as to the number of different oligosaccharide chains in each of the domains, but we do not yet know which one is the N-termininus.

Along with these separation experiments we can solve the one problem just alluded to; separation of the protease-resistant domains, will permit us, by use of the gas-phase micro sequencer, now available in this department, to determine which one represents which end of the epo molecule, since we already know that native epo has an N-terminal alanine.

We will then determine the monosaccharide composition of the isolated oligosaccharide chains by the same micro method used for the overall composition studies[10] (see preprint by Dordal, Wang and Goldwasser in appendix). It would be very interesting to learn the sequence of monosaccharides in each oligosaccharide. Once again, if commercial production via the cloned human gene or if IW32 cells can provide the needed quantity we could use the mass spectrometer-gas-liquid chromatographic technique used for orosomucoid.[32] Until the requisite amount for that method becomes available, we plan to use an alternative method based on enzymic hydrolysis.[33] There are available specific exoglycosidases which can act on α and β linked sialyl, galactosyl, N-acetylglucosaminyl, mannosyl and fucosyl residues. Since we will be hydrolyzing individual aparagine-oligosaccharides, the contamination of exoglycosidase by proteases will not be a problem. The specificity of each exoglycosidase will be tested with the appropriate p-nitrophenyl glycosides of N-acetylglucosamine, galactose, mannose, and fucose, i.e., one enzyme should act on only the one glycoside, if it acts on more than one, further purification, by reverse-phase HPLC, will be carried out. We plan to use these enzymes and to use the GLC method, already metnioned,[10] to determine which enzyme can release a specific monosaccharide from the terminus of the oligosaccharide. By these methods we should be able to arrive at a provisional assignment of the monosaccharide sequence for each separable oligosaccharide.

Labeling of epo

In the study of epo binding we have been faced with an experimental barrier; iodinated epo is inactive; ^{14}tritiated epo has a specific activity that makes it feasible to study binding only with cell populations enriched for epo-responsive cells. Many additional binding experiments of interest could be performed if an epo preparation with specific activity characteristic of a short-lived isotope such as ^{125}I, ^{35}S or ^{32}P could be made, with biological activity intact. We propose to continue our efforts to prepare such an epo derivative. To date, no method we have investigated has yielded an active enough preparation. There are, however, several approaches that are still worth trying.

There are about four tyrosine residues in native epo, only one of which is accessible to iodinating reagents; the one that is in the active region. We know that epo can be denatured and renatured with little or no loss of biological activity.[11] We prose to denature epo with 6 M guanidine, iodinate with ^{125}I and renature. There will be approximately a 25% chance of modifying the "active" tyrosine, therefore about 75% of the original activity should be regained on renaturation, if one, or more, of