EXHIBIT 3
Part 3a of 5

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR OR AWARD CANDIDATE (Last, first, middle)	SOCIAL SECURITY NUMBER
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PROGRESS REPORT

Our progress over the past 4 years will be reported in a rather truncated fashion and will emphasize positive results; details can be found in the appendix containing published papers and preprints of those in press or submitted for publication.

Methods of purification of epo In order to use the still limited amounts of crude available epo most efficiently, we have devoted considered effort to simplify the method and improve the yield. One of the initial and important steps in purification is the inactivation of degradative enzymes (proteases and sialidases) in the urine concentrate. Our published method[7] was lengthy (7 days) and resulted in about 37% loss of activity. By precipitating epo from the phenol solution with 90% ethanol we have shortened the time to 1-2 days and improved the yield to 82%. The next step in the method, ethanol fractionation,[1] was simplified (without increased yield) by precipatating epo from a solution containing 6 M guanidine and 10 M LiCl. The 75-90% alcohol precipitate under these conditions is completely soluble, in contrast to the older method, and does not need re-extraction.

The most costly step (with respect to yield) in the past has been chromatography on sulfopropyl Sephadex. This procedure is important for subsequent purification and we have not found a suitable alternative. It has been modified, however, by inclusion of ethylene glycol in the eluting buffer and the yield increased from 55% to 90-100% with an increase in purification factor from 6 to 11.

We have adopted the wheat germ agglutinin (WGA) method, described by Spivak et al[8], as a standard part of the procedure, but have used more easily available oligoacetylglucosamine as the eluent instead of costly chitobiose. The oligo glcNAc is prepared by partial hydrolysis of commercial chitin. The WGA step now has a purification factor of 3-4 and a yield of 90-100%. The epo derived from this step (after going through the earlier steps) is essentially pure; by potency (≤70,000 u/AU) and SDS gel electrophoresis (single band). The lectin column, however, does not separate the α and β forms if such separation is needed (e.g.for studies of oligosaccharide structure), the hydroxylapatite step must be used with some unavoidable loss.

Our studies of purification methods have been greatly facilitated by using pure epo labeled with ^{3}H by the Van Lenten and Ashwell[9] method as a tracer, obviating the need for assays of every fraction collected. Our current method is summarized in Table 1 (appendix).

One potentially important purification method is still not satisfactory; we have spent much time on the possible use of monoclonal anti-epo (see below) for immunoaffinity chromatography. Our results have not been uniformly successful.

In one, non-typical, experiment the antibody column was used to purify an alcohol preciptate (75-90%). Input was 55,680 units at a potency of 1780 u/AU (2.5% pure), 32.8% of the activity was in the non-adsorbed fraction and 64.4% (35,489 u) was eluted with SCN^{-}, Ca^{++}, ethylene glycol. The potency was 35,480 u/AU (51% pure) and the purification factor was 20. If we can learn how to get this kind of result routinely, this method could eliminate much processing.

Chemical properties The carbohydrate composition of both α and β epo were determined by a micro modification of conventional gas-liquid chromatography.[10] The results show that the two forms differ significantly in N-acetylglucosamine and N-acetylneuraminic acid. The oligosaccharide chains were found to be N-linked, as demonstrated by loss of labeled sialic acid after treatment with endoglycosidase F (specific for asparagine-linked complex oligosaccharides). This enzyme caused the conversion of epo with a apparent molecular weight of 34,000 to a product with molecular weight of 22,000. It is worth noting here that our original estimate of the molecular weight of native epo as 39,000 was inaccurate. More recent measurements, including the calculation of partial specific volume from the newly determined carbohydrate composition ($\bar{v}$=0.67) along with s=3.03 S from sedimentation velocity, in the separation cell, and F=32.4 Å, from a gel permeation column, indicate M to be about 34,000, in good agreement with the value derived from SDS gel electrophoresis. The carbohydrate portion of epo can also be removed

with protease-free mixed exoglycosidases (generously provided by Dr. R. Hill, Duke Univ.) which also causes a decrease in apparent molecular size of about 27%.

Treatment with endo F causes a loss of about 40% of the biological activity, when assayed in vitro and 53% when assayed by RIA. There is extensive aggregation of the deglycosylated epo which may account for the fall in activity; when deglycosylated epo is in the presence of detergent, however, it is monomeric. The same is true of epo treated with exoglycosidases; the size in detergent is smaller than native epo, but on the HPLC sizing column, we found it to be rapidly and extensively aggregated, with a decrease in biological activity in vitro. When assayed in vivo, deglycosylated epo is devoid of activity.

Our studies of which features of the epo molecule are essential for its striking biological activity revealed the following: the carbohydrate is not required, at least down to the asparagine-linked N-acetylglucosamine. There is at least one tyrosine residue, easily available for iodination in native epo, that is required for activity since any method of iodination that involve tyrosines results in inactivation: there is free amino group (α or ϵ) required since addition of the Bolton-Hunter reagent causes inactivation. Previously it has been shown that tryptophan residue is probably involved in the active site.

We have shown by partial proteolysis of native epo that it consists of three domains:[11] the N and C terminal regions each with a molecular size of about 16,000 (rough estimate) and which have a compact, protease-resistant, structure, connected by a smaller, more loosely structured region that is sensitive to trypsin, chymotrypsin, V-8 protease, pepsin and endoproteinase Lys-C, but not to endoproteinase Arg-C. This connecting region probably contains the active site, since biological activity is lost upon proteolytic cleavage. In addition, if an antibody capable of neutralizing the biological activity, and hence reacting with the active site, is complexed with epo, before exposure to trypsin, there is no proteolysis. An indifferent immunoglobulin has no such protective action.

All of the label after iodination is localized in the connecting region, indicating that the accessible tyrosine is in the active site. All of the ^3H label, associated with the carbohydrate, is found in the larger domains suggesting that the active region contains no oligosaccharide. The different effects of the two endoproteinases indicate that the connecting region and active site contain a lysine residue, which probably has the amino group that reacts with the Bolton-Hunter reagent.

By use of labeled N-ethyl malemide and iodoacetic acid we demonstrated that epo contains four sulfhydryls in two internal disulfide bonds. The disulfide cannot be reduced with dithiothreotol unless epo is first denatured and cannot be alkylated unless epo is denatured. Alkylation of denatured and reduced epo results in complete loss of biological activity. If, however, denaturation and reduction are followed by oxidation and renaturation, about 85% of the biological activity is found. This suggests that, in addition to the amino acids mentioned earlier, we must include the two disulfide bridges as being required for activity. They may be needed for the establishment of the proper conformation to permit the connecting region to interact with specific receptors, or may have secondary sites for interaction with receptors.

Monoclonal anti-epo We have developed a rat:mouse hybridoma that secretes a monoclonal antibody specifically directed against epo.[12] It required screening about 3000 rat:mouse hybrid cells to get one stable clone. The properties of the antibody are consistent with it being specific for epo, but it is non-neutralizing. The use of this antibody for immunoaffinity purification has already been discussed. We have also used it to study binding of epo.

Binding of epo to target cells Interaction of epo with potential target cells were studied by use of the monoclonal antibody labeled with biotin and added to marrow cells after addition of epo.[13] The cells were then exposed to fluorescent avidin and, after washing, photographs of many microscopic fields were taken by epi-fluorescence. Dead cells were determined by propidium iodide uptake (approximately 0.4% of the total) and the fraction of epo positive cells refers only to living cells. By this method we

showed that 1.3±0.4% of the total nucleated mouse marrow cells have detectable fluorescent signals. Another method of fluorescent labeling was also used. We prepared a fluorescent derivation of epo using N-7 dimethylaminomethylcoumarinyl maleimide (DACM). This compound is generally used to form a thioether with a sulfhydryl group, thereby generating a fluorescent compound. In the case of epo, the reaction appears not to be with a thiol, but there is still a covalently linked, fluorescent epo formed. Using this DACM-epo we measured the frequency of epo-binding cells by the same method used for the indirect immunofluorescence. With rat marrow we found that 1.4±0.4% of the cells bound epo specifically. In addition, using physiological perturbations of the rat, we found the expected changes in this frequency. It is of some interest that the frequency of epo-binding cells in normal marrow is 2-3 times greater than the frequency of CFU-E and suggests that cells later than CFU-E still possess receptors. Neither mature red cells, thymocytes, Friend cells nor K562 showed any specific binding of epo, as measured by fluorescent positive cells..

Quantitative study of epo receptors on hemopoietic cells has been made difficult by two factors: .1) the low incidence of target cells in normal hemopoietic tissue and the difficulty in routine purification of such cells from marrow and 2) the loss of biological activity when epo is iodinated,[14] eliminating a facile method for measuring binding. It should be noted with respect to the former, that the pure CFU-E isolated by the method of Nijhof and Wirenga[15] come from mice that have high circulating epo and seem to have their receptors largely saturated. No binding to such cells has yet been detected.

We used an alternative approach. In mice infected with the anemia strain of the Friend virus the spleen contains a large proportion of cells that require epo for terminal differentiation to red cells.[16] In colonies grown from such spleen cells the proportion of epo-dependent cells is close to 100%. We have used these cells and ^{3}H-epo (see above) to study binding quantitatively. Our data show a single class of specific epo receptors with a K_D of 5.2 nM and a mean number of receptors per cell of 660. Since these cells are maximally stimulated into hemoglobin synthesis at about 0.06 nM, there is the strong inference that a very few (as few as 8) epo molecules per cell is sufficient for the effect. At less than maximal stimulation, of course, the number would be even less, suggesting that even one molecule per cell has an effect. If this is a general phenomenon (.i.e.,also the case for normal epo-responsive cells) it is going to make further study of the intracellular effect of epo difficult indeed.

Effect of epo on membrane synthesis Since erythroid differentiation results in a cell with a specific set of membrane proteins, and since red cell membranes are among the best characterized, we studied the effect of epo on relatively early precursor cells (pre-CFU-E) with respect to the biosynthesis of the characteristic membrane components[17] In these cells, in culture, stimulated hemoglobin synthesis started later than 24 hours after addition of epo and was maximal at 96 hours. Synthesis of the major membrane glycoprotein constituent, glycophorin, was maximal at 30 hours; significantly later than hemoglobin. In contrast, Band 3, the major integral membrane protein was detectably present at 18 hours and maximal at 66 hours, suggesting that "remodeling" of the exterior of the cell started prior to the onset of hemoglobin synthesis. In addition epo had a significant effect on the synthesis of some membrane proteins not found in the mature red cell, but present in the precursor cells. Another important membrane component, spectrin, was present from the start of incubation and did not change much in the early phases of epo action, indicating that it was characteristic of early hemopoietic cells as well as red cells. These findings imply that in the cascade of molecular events that occur between the initial effect of epo and induced hemoglobin synthesis, there may be an effect on membrane protein formation that is obligatory for the remainder of the differentiation process. It could be something like increased expression of the transferrin recptor.

Application of RIA The availability of pure epo, prepared for the first time in this lab,[1] permitted us to develop an RIA which has been used in several types of study.[2] An early one was to determine the normal circulating titer. If the blood is clotted

at ice bath temperature, the normal serum epo level is 21±6 mu/ml (normally distributed) with no difference between males and females.[18] If it is clotted at room temperature there is loss of about 30% of the epo, yielding a value for normals of 15 mu/ml. Patients with primary polycythemia (untreated polycythemia vera) have a mean serum titer of 15 mu/ml (room temperature clotting), and those with polycythemias secondary to a variety of disease states (heart, disease, lung disease, cancer) have a mean titer of 90 mu/ml. Use of a cut-off value of 30 mu/ml permits the correct diagnosis of primary vs. secondary polycythemia in 94% of the cases.[19]

Using the epo RIA to determine the serum titer in patients with chronic renal disease we found a discrepancy between that found by bioassay and RIA; the latter being considerably higher.[2] Further study of this showed that the sera of these patients contained immunoreactive components of smaller size than native epo.[20] The presence of these "fragments" may account for the discrepancy, if they have no biological activity. This is still to be determined.

In collaborative studies of clinical problems we found that erythrocytosis in "end-stage" renal disease, secondary to glomerulonephritis, is probably a result of increased erythropoietin production.[21] In an analogous study our assay method helped establish the finding that there is a genetic erythrocytosis, an autosomal dominant, characterized by autonomous erythropoietin production.[22]

We have also used the RIA to study the epo titers of patients with rheumatoid arthritis (in collaboration with several clinical colleagues) and found that most of those with moderate anemias do not respond with an increase in epo titer.

In a very limited clinical trial of the effect of administered pure human epo, in correcting the anemia of chronic renal disease (see below) we also measured the clearance rate in three patients and in two normal volunteers by RIA. In the patients (Fig 1, appendix) we found that after the initial rapid equilibration ($t_{1/2}$ approx 4-17 min) there was, in two cases a slower clearance rate ($t_{1/2}$ 60-140 min) followed in all three by a secondary reappearance of immunoreactive material in the circulation, which then decayed off much more slowly. Examination of the immunoreactive material on sizing columns showed that during the first phase (exponential clearance) it was largely the size of native epo (34K); the material found in the secondary rise phase was for the most part the size of cytochrome (14K) or smaller. This suggests that in these patients there is a tissue degradative process which release some "fragments" that still contain an antigenic site recognized by the antibody we used. These "fragments" may be similar to those found in the sera of similar patients not treated with epo. In the normal volunteers (Fig 2, appendix), the initial phase had a $t_{1/2}$ of 10-20 minutes, probably representing equilibration time and an approximate half-time for clearance of 1.8-2.6 hours. There was either a slight secondary (Fig 3, appendix) rise at about 90 minutes or a long plateau, suggesting that "resecretion" may have occurred but was less prominent than in the patients on dialysis. These data are very preliminary but indicate the effective use of the RIA in clinical studies. The effect of epo on the patients will be summarized below.

Since we have an antibody that recognizes rat epo we could, (in collaboration with Drs. S.M. Hopfer & F.W. Sunderman, University of Connecticut), analyse the effect on epo titer of intrarenal Ni_3S_2, which can induce renal tumors, and polycythemia. We found that serum epo was significantly increased, peaking at 3 weeks after the injection.[23] At this time, the rat kidneys contained an average of almost 1 u/g of extractable epo; about 10 times the amount found in control kidneys. There are indications that some of the renal immunoreactive material may be a biologically inactive precursor.

Metabolism of epo We used ^{125}I-epo, despite its lack of biological activity, to trace the fate of epo injected intravenously into intact rats.[24]

During the steady-state, the metabolic clearance was 256±7 µl/min/kg/; 19±2 µl min/kg (7.4%) of which could be accounted for by excretion in the urine. Urinary clearance was less than 0.3% of the glomerular filtration rate. The plasma half-life (from both pulse injection and constant infusion experiments) was described by a single