EXHIBIT 2
Part 2 of 3

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Goldwasser, Eugene	494-14-6535

Objective: We plan to prepare enough pure human erythropoietin (epo) to: a) finish the determination of the primary structure of the polypeptide chain, b) start on an enzymic method of determining the sequence of the sugars in the oligosaccharide chains once we have methods for their separation, c) study the structural requirements for the biological activity of epo and d) continue the study of its clinical efficacy and physiological properties in vivo. In addition, we will try to develop additional monoclonal antibodies to epo with the intent of studying different domains against which the antibodies may be directed. The existence of naturally occurring immunoreactive fragments of epo in serum and urine makes it possible to isolate them in pure state and use them, as well, for additional studies of biological and immunological activities.
Review: Since the last competitive renewal of this grant there has been substantial progress in in this field. With the use of pure epo, (1) supplied from this laboratory, radioimmunoassay (RIA) was made possible (2,3) and is now operating on a fairly routine basis in three other institutions. The results with the RIA have largely confirmed the basic physiology of epo as determined by more cumbersome and less sensitive bioassays. (2,4). Additionally the RIA has permitted the determination of the normal serum titer of epo (5) and has been shown to provide a means of distinguishing between primary and secondary polycythemia (6). The existence of immunoreactive fragments of epo, presumably without biological activity, in sera of patients with chronic renal disease has been shown in two laboratories (3,7). Success in developing a hybridoma that forms a monoclonal anti-epo was reported from two laboratories (8,9). One of these antibodies is non-neutralizing, the others appear to be neutralizing.

There have been several reports of assays for epo by cultured cells (10-15), generally based on the original observations made in this lab in 1963 (16). There have also been several studies of circulating epo in disease states, for the most part using bioassay methods (17-21). A comparison between the bioassay and the hemagglutination-inhibition method showed the latter to be wanting (22). There have been several important additions to techniques for purifying epo reported (23-27). Two short papers on some chemical properties of epo in the crude state have appeared (28-29), and two on epo producing tumor cells (30,31). Schooley showed that an $F(ab')_2$ fragment derived from a neutralizing anti-epo, formed a complex with epo that was biologically active (32).

Progress report: In the three years since this project was last reviewed progress has been, in my view, substantial. With respect to the chemistry of epo we now know that the assumption of purity, based on chromatography and gel electrophoresis, was a valid one. In collaboration with Dr. Leroy Hood (California Institute of Technology) we have started the study of the amino acid sequence of both the α and β forms epo using the micro-solid-state sequencing method developed there. We find that both α and β epo have a single N terminal residue, alanine, and that the sequences of the next 27 residues are identical for the two forms. This suggests that the protein portions of the two may be identical, although differences based on amidation have not yet been ruled out.

Because of discrepancies in the amounts of carbohydrate and protein found by analytical techniques, we reinvestigated the molecular weight of epo. The previously reported value, 39000, was based on a sedimentation coefficient(s) of 3 S and a Stokes' radius ($\bar{r}$) of 32.5 Å. This value for M agreed with that found by gel electrophoresis in the presence of dodecylsulfate and mercaptoethanol. Since the value by electrophoresis seemed to be independent of gel concentration (over a rather narrow range) we provisionally accepted the value of 39 K. The value of M derived from s and $\bar{r}$ is dependent on the partial specific volume and using a tentative value of 40% for the carbohydrate content a value of $\bar{v}$=0.67 can be calculated. This results in M=34000 which is fair agreement with that found by the sum of the protein and carbohydrate contents.

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We have made reasonable progress in our study of the carbohydrate composition of epo, but have not yet, because of the dearth of material, established the number of oligosaccharide chains. By scaling down the gas-liquid chromatographic method for determination of trifluoroacetyl derivatives of the monosaccharides, to permit quantitative determination of about 1 nmole, we have determined the carbohydrate composition of the α and β forms and found them to be significantly differently with respect to sialic acid and N-acetylglucosamine.

MONOSACCHARIDE COMPOSITION OF ERYTHROPOIETIN(residues/mole)

	α	β
Fucose	4.3	3.8
Galactose	11.7	10.1
Mannose	6.8	7.1
Glucose	0.7	0.7
N-acetylglucosamine	11.8	8.1
Sialic Acid	15.8	11.5
N-acetylgalactosamine	0	0

In addition, we have used two methods of removing carbohydrate from the glycoprotein: solvolysis with pyridine in anhydrous HF and by use of a preparation of mixed glycosidases (generously provided by Dr. R. Hill). In both cases, approximately 80% of the carbohydrate was removed; the only remaining sugar being N-acetylglucosamine attached to the protein. Biological activity, to the extent of about 80% was retained by the aglycoepo, as measured by the in vitro method. We have not yet done the in vivo assay. These findings suggest, strongly, that the carbohydrate is not required for the interaction of epo with its target cells, even though it might be required for in vivo "survival." They also make our longer range goal of cloning the epo gene and expressing its synthesis by bacterial cells a feasible one.

Over the past few years, since we found that labeling epo with ^{125}I either on the tyrosine residues on the free amino groups caused inactivation of its biological activity, we have been attempting to use the sulfhydryl groups of epo as a functionality for alkylalation with an iodinatable reagent. (The tritiation of epo on the carbohydrate is feasible and yields an active derivative but of too low specific activity to use for binding studies). The basis for our experiments was our finding that N-ethylmaleimide (NEM) reacts with epo to the extend of 3 moles/mole without loss of biological activity. This observation, it now appears, was artifactual; the NEM was not covalently bound. Our more recent data now clearly show that the SH groups are not accessible to the reagent unless epo is denatured and reduced. Under such conditions it reacts with NEM (4 moles/mole). The S-alkylated epo is devoid of biological activity, suggesting that two disulfide bonds are required. These findings make it apparent that our attempts to label with ^{125}I via the cysteine residues were futile.

There was, however, an interesting paradox revealed. The fluorescent SH reagent. (N-dimethylamino-4-coumarinyl)-maleimide reacts with epo to generate a derivative that is fluorescent and has full biological activity. Despite the fact that the product is not the epo thioether we first assumed, and even though its chemical nature is not at all clear, we have used it to study epo responsive cells. The frequency of these cells in marrow; about 1.4%,is the same using the unknown epo derivative as it is using biotin-labeled monoclonal anti-epo and fluorescein-labeled avidin.

As just indicated, we have succeeded in developing a hybridoma that forms monoclonal anti-epo. We screened about 3000 rat-mouse hybrids before we found one stable clone. The screening was done by a binding assay using ^{125}I-epo; the resulting antibody does not neutralize the biological activity. We have begun to accumulate purified monoclonal anti-epo IgG from the peritoneal ascites fluid of nude mice

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carrying the hybridoma and to develop an immunoaffinity method to purify epo. In regard to other epo purification techniques we have made a significant, if small, change in the published method that improves the yield considerably. The chromatographic step on sulfopropylsephadex in which we formerly had a yield of only 50% was improved by adding ethylene glycol to eluting buffer, to a final concentration of 20%. Under these conditions, the yield is 100%.

The use of the RIA and gel permeation chromatography permitted us to show that, in sera from patients with chronic renal disease, the most probable explanation for the discrepancy in epo titer between the RIA and the bioassay is the presence of immunoreactive "fragments" smaller than cytochrome-C. We find a similar situation in following the clearance of epo injected into patients on dialysis. There is a rapid loss of RIA titer from the circulation, followed by a secondary rise, in all three patients studied. Analysis of the molecular size of the immunoreactive material showed that in the rapid disappearance phase of the clearance curve there was about 75% native-sized epo and 25% as small fragments; in the secondary rise part of the curve there was about 25% native and 75% fragments. These data indicate that epo, in patients with chronic renal disease, is broken down after it is cleared and the breakdown product is released back into the circulation. We have also found evidence for small immunoreactive fragments in urine from patients with aplastic anemia.

Lastly, because of the great need for material for research in this field, we have supplied to NIH for distribution, two batches of epo. The first, for RIA purposes, is pure α form at 70,000 units/mg protein and we have given 95 vials at 57 U/vial to be allocated by a subcommittee. The second preparation is only 1100 U/mg of protein but has been freed of colony-stimulating factor, endotoxin and burst-promoting activity. It is non-inhibitory for mouse burst cultures up to 10 U/ml and also supports the growth of human bursts. We have provided 305,660 units, in aliquots of 1700 units, for distribution.

Publications:

JF Garcia, JB Sherwood and E Goldwasser Radioimmunoassay of erythropoietin. Blood Cells 5, 405-419 (1979)

JB Sherwood and E Goldwasser A radioimmunoassay for erythropoietin. Blood 54, 885-893 (1979)

T Terasawa, M Ogawa, PN Porter, DW Golde and E Goldwasser Effect of burst-promoting activity (BPA) and erythropoietin on hemoglobin biosynthesis in culture. Blood 56, 1106-1110 (1982)

HP Koeffler and E Goldwasser Erythropoietin radioimmunassay in evaluating patients with polycythemia. Ann Int Med 94, 44-47, (1981)

TL Weiss and E Goldwasser The biological properties of endotoxin-free human erythropoietin. Biochem J 198, 17-21 (1981)

E Goldwasser Erythropoietin and red cell differentiation in Control of Cellular Divsiion and Development 1981 pp 487-494 Eds. D Cunningham, E Goldwasser, D Watson, CF Fox, (AR Liss, NY)

BD Tong and E Goldwasser The formation of erythrocyte membrane proteins during erythropoietin induced differentiation. J Biol Chem 256, 12666-12672 (1981)

In press

CW Distelhorst, DS Wagner, E Goldwasser and JW Adamson Autosomal dominant familial erythrocytosis due to autonomous erythropoietin production.

TL Weiss, CJ Kavinsky and E Goldwasser Characterization of a monoclonal antibody to human erythropoietin.

Submitted

JB Sherwood and E Goldwasser The heterogeneity of immunoreactive human serum erythropoietin.

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Methods:

Because this is a request for a supplement, this section of the proposal will be in less detail than would be the case for a full grant.

By using the monoclonal antibody affinity column, already tested, and one or two other steps we plan to prepare as much pure epo as possible from the remaining material we have in stock. Now that the supply from Japan is not reliable, we would also be glad to arrange a cooperative mechanism by which we return one-half of the final yield of pure epo to a lab that provides a source of active urine. We estimate that the material now on hand is sufficient for about 5-6 years of the research discussed below, so we will not be hampered by scarcity. This state is aided by our success in improving the yield at the sulfopropyl Sephadex step.

In our continued search for alternative sources of human epo, we will use the affinity column to concentrate the epo in fractions of plasma prepared by the Red Cross. This is some indication that there may be a significant, small amount in one of the fractions that is routinely discarded. If we can find a simple method of recovering these small amounts, the many thousands of liters of plasma may be a secondary source.

We will continue our collaboration with Prof. Hood in order to get as much sequence information as possible. This sequence will then be used in several different types of research: for studies of structure-function relationships, to prepare fragments of known structure by limited proteolysis (e.g. trypsin and chymotrypsin at very low concentration, short time and low temperature, V8 protease) or by chemical cleavage methods at specific residues, such as methonine, tryptophan and perhaps cysteine. In this last case we have already found that we can prepare such fragments, but since the N termini are blocked we cannot use them for sequencing. The fragments may be useful for study of specificity of interaction with antibodies, but that will have to wait until we have the sequence of the whole polypeptide so we know the structure of the fragments broken at the cysteines.

The sequence information will also be used to prepare a synthetic oligodeoxy nucleotide probe, following the method of Agarwal, for the eventual purpose of isolating the epo mRNA as an essential prerequisite to preparing the cDNA that can be cloned. This last aspect is still some time away.

We are also going to use pure epo labeled with ^{3}H in the terminal sialic acids, to isolate, by HPLC, the oligosaccharide chains, after pronase digestion of epo. Once this is accomplished, we will use stepwise hydrolysis by specific glycosidases for structure studies. After each enzyme is used we will determine the released monosaccharide, if any, by gas-liquid chromatography techniques already developed for the composition studies. This method involves "guessing" which glycosidase to use at which step but is still feasible, whereas the NMR technique used for orosomucoid would require much more epo than we can produce within the next several years.

As we continue to study the structure of human epo, we will use the affinity column method for the purification of rabbit epo. Over the past years we have accumulated many liters of active rabbit serum that are stored frozen, waiting for this method. If our monoclonal antibody does not react with rabbit epo (not yet tested) we will continue to produce similar antibodies until we find one that does cross react. There is reason to believe that such cross reaction will not be too difficult to find since we have tested a rabbit anti-human epo and found it to react with both sheep and mouse epo. Pure rabbit epo, once we convinced of its homogeneity, will be sequenced in order to determine whether there are homologous structural features in the two species and whether those features are involved in biological activity.

The presently available monoclonal antibody is non-neutralizing. We will prepare other antibodies, in the search for one that neutralizes, and to accumulate antibodies directed at as many different domains of the epo molecule as possible. The antibodies will be produced, either by the rat-mouse hybrid method that has already been successful or by in vitro immunization. The non-neutralizing antibody can

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be used to label epo for binding studies. We will prepare the Fab fragment, in order to avoid problems with Fc receptors, label it with ^{125}I, make the Fab-epo complex and use that to study quantitative binding by marrow and other potentially epo-responsive cells. Labeling with fluorescein and/or rhodamine will also be used in order to identify, by microscopy, the cells with epo receptors. This will be an important part of our effort in the immediate future. Among the questions that need exploration by these methods are: a) since CFU-E can be purified to a great extent, what is the number of receptors for epo on these late erythroid progenitors? b) how does that compare with the number per BFU-E? These latter cells cannot, yet, be purified to any great extent, but we can prepare marrow cell populations almost devoid of CFU-E by keeping mice plethoric for some time; the number of BFU-E is not appreciably changed. By counting the number (frequency) of labeled cells in such a population and by direct binding studies of labeled epo-Fab we can determine the mean number of binding sites per cell. These should represent pre CFU-E, including BFU-E; the latter can, of course, be estimated to a rough extent by burst counting. We also plan to test a variety of erythroid-like cell lines such as FLC, K562, and HEL for epo binding.

The monoclonal anti-epo will also be used to develop a solid-state RNA based on the specificity of the antibody, which can be "grown" in large amount rather than on the, still rare, antigen. This method, if the volumes and amounts can be adjusted, could be 4-5 x more sensitive than the existing RIA, especially since the label (^{125}I) will be on a commercially available reagent, protein A. We can thus use more label and more reagent to be labeled. We need a more sensitive (and would like a more rapid) assay, in order to study the fragments of epo found in serum and to determine whether fragmentation is a general, non-pathological, phenomenon, or restricted to certain disease states. Similarly a more sensitive assay will be of great help in the study of clearance rates in laboratory animals.

Although we have screened eight human renal tumors in the past year we have not yet found one that stably secretes spo in significant amounts; two of the eight did show a transient production of epo which decreased within a few days. None of the tumors was from a patient with erythrocytosis. We intend to continue this screening and, for those cells that can be established in culture to study the effects of methods known to increase epo production in vivo; these include hypoxia, colbalt, cyclic AmP, prostaglandin, testosterone and combinations of them. These tumor cells, if they can be shown to either produce epo in vitro or contain a large amount, can be used as sources of epo mRNA as indicated above.

Last to be mentioned in this brief account of our experimental aims, is the accumlation of enough pure epo to do a significant clinical trial of its effect in the correction of the anemia of chronic renal disease, and possibly of inflamatory diseases. Preliminary experiments, indicate that epo may well be useful in renal disease patients. When enough material is at hand we will continue these trials and when possible we will initiate collaborative studies in other institutions.

Justification for supplemental funds: The laboratory staff consists of two professional biochemsits, three technicians, a lab helper and two graduate students who are not being paid by this grant. The kinds of problems we are studying are expensive since they involve costly HPLC columns, ultra pure reagents and solvents, expensive animals, animal care and media for cell culturing. Despite our realization that funds are limited and our careful monitoring of each expenditure, we have found, in the last two years, that the non-personnel budget simply does not provide enough funds for our work. Some of the funds restricted, by the study section; for epo purchase were made available to us during the past two years for general purposes, because the Japanese source of epo is now very uncertain. This special lifting of restriction permitted us to carry out our experimentation for the full year. Without those funds we would have been in the position of being able to do nothing but read and write for the last 2-3 months of the budget year. In part this "shortfall" is due to increases

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in expenditure that were not calculable when the original proposal was submitted. These are due to mandatory raises for the non-professional personnel, to extraordinary increases in animal care costs and to the shift to important new, but expensive, techniques such as HPLC.

I realize that this is already a large grant, but the size is not going to be materially changed since the bulk of the funds (78%) is used for personnel costs. There is added to this request, a small addition to the personnel category, that of the instrument designer for 2.5 hours/week. This represents part of the cost to the department of maintaining the machine shop. We do make considerable use of the services of the machine shop to service equipment not under maintenance contract and to construct items that are not commercially available such as micro gel electrophoresis apparatus which we now use routinely.

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