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"IN VIVO STUDIES OF HEMATOPOIETIC GROWTH FACTORS FOR USE IN MILITARY PERSONNEL."

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13. ABSTRACT *(Maximum 200 words)*

The secondary structures and thermal denaturation/renaturation of recombinant human erythropoietins were compared using UV circular dichroism. Marked differences were observed between proteins expressed by different host cell lines, presumably due to dissimilar glycosylation patterns. A pharmacokinetic study of monomeric erythropoietin carried out in baboons demonstrated a very short plasma half-life of three hours. The proper formulation of the protein for these studies was established. Work on erythropoietin +dimers progressed, but high yields in each reaction were different to achieve. Knowledge of the differing glycosylation patterns will help improve these yields. Work on erythroid colony stimulation factor was resumed, and rodent bone marrow cultures were established.

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MILESTONES

This proposed research consists of the following milestones.

1. Long-acting erythropoietin
   Recombinant human erythropoietin - production, purification and characterization.
   Rodent studies
   Primate studies
   In vitro studies

2. High potency erythropoietin - work temporarily suspended; see budget reallocation
   Rodents
   Primates
   In vitro studies

3. Erythroid colony stimulating factor - work suspended; see budget reallocation
   Rodent studies
   Primate studies

1. Long acting erythropoietin.

Significant progress has been made in our proposed research on long-acting erythropoietin. This progress has occurred in the areas of characterization of recombinant human erythropoietin, primate studies of long-acting erythropoietin and in vitro studies.

With respect to characterization of recombinantly produced human erythropoietin, important facts with regard to protein structure have emerged. We have initiated a collaboration with Dr. Mary Walsh, Biophysics Department, Boston University School of Medicine. Dr. Walsh is a renowned expert in spectroscopic evaluation of protein structure, especially regarding glycoproteins and the effect of oligosaccharides on polypeptide folding and conformation. We have undertaken a series of circular dichroism experiments with Dr. Walsh and have compared the far UV circular dichroic spectra of recombinant human erythropoietin produced in Chinese hamster ovary cells (CHO) and with that produced in baby hamster kidney cells (BHK). The erythropoietin produced in baby hamster kidney cells was produced according to the methods outlined in our proposed research. The far UV CD spectrum reflects the secondary structure of a protein, that is, the structures known as alpha helix, beta sheet and random coil. These structures within a protein bear directly upon the relationships of the individual peptide bonds within the protein. We have discovered a significant difference in the far UV CD spectra of CHO and BHK derived recombinant human erythropoietin, indicating a pronounced structural difference. Since the amino acid sequences of these two proteins are identical, the structural difference must be attributed to the oligosaccharides that are attached to the protein during postranslational

processing in the cell. This finding could explain the pharmacological differences that have been reported for these two types of recombinant human erythropoietin. Furthermore, preliminary studies using a heat denaturation protocol (which results in unfolding of the protein followed by the return to room temperature allowing protein refolding) demonstrated that BHK and CHO recombinant erythropoietins exhibit different kinetics of unfolding and refolding. Perhaps most importantly, the proteins refold to their original CD spectra. This indicates that the oligo saccharides attached to the protein appear to be responsible for inducing protein folding and for the final secondary structure of the polypeptide chain. Such a finding is quite novel in the field of protein structure and bears heavily on the so called "protein folding problem" that is of such great importance in all fields of biochemistry. Since it is known that not all molecules of recombinant human erythropoietin produced by a single cell type have the same carbohydrate structure, but rather, differ in subtle but important ways from each other, we hypothesize that even within a preparation of recombinant Epo from a particular cell type, structural differences exist among the individual Epo molecules present therein. This has important bearing on our studies of the production of Epo-Epo dimers (long-acting Epo) and our studies of high-potency erythropoietin (see below).

Our second pharmocokinetic study of monomeric recombinant human erythropoietin in baboons was very successful. Highly purified protein was formulated in a solution of phosphate buffered saline containing 5% human serum albumin. This formulation was agreed upon after discussion with our collaborator, Dr. C. Robert Valeri, and his staff at the Naval Blood Research Laboratory. Two samples of material were prepared and two baboons were injected intravenously with a total volume of 5 ml each. At specified times thereafter, blood samples were drawn, plasma was prepared and transported back to our laboratory for determination of circulating erythropoietin levels by radioimmunoassay. Based upon the weight of these animals, we had calculated an approximate plasma level that would be achieved after complete mixing of the erythropoietin in the circulation prior to significant loss due to physiologic clearing from the circulation. Both animals achieved close to 90% of the theoretical maximum, thus indicating that the formulation employed was very successful in delivering the recombinant protein to the circulation of the animal. The pharmacokinetic decay curves for each of the two animals were very similar, resulting in a plasma half-life of approximately three hours in each. We are very confident that this formulation will allow us to achieve a high degree of precision in determining the plasma half-life of dimeric forms of long-acting erythropoietin. We judge the formulation problem to have been solved.

As reported previously, we have continued to pursue our in vitro studies to optimize production of Epo dimers. Our yields are still lower than expected. However, the studies detailed above on the role of oligosaccharides in erythropoietin structure have given us some clues to the explanation for this problem. It is clear that oligosaccharides affect folding of the polypeptide backbone. It is also clear that recombinant erythropoietin, regardless of which cell type it is produced in, exhibits pronounced microheterogeneity due to different oligosaccharide structures attached to each individual molecule. Therefore, we hypothesize that there is significant molecule to molecule variation in structure within a preparation of recombinant human

erythropoietin. This molecule to molecule variation in structure results in a differing degree of derivatization with our chemical crosslinkers within the pool of molecules. This results in a less than ideal yield of dimers. Current technology in engineering of cells producing recombinant proteins still does not allow us to express identical glycoprotein molecules. This microheterogeneity will have to be lived with. Certain possibilities exist which may allow us to improve on this situation. For example, we may be able to enzymatically "trim" the oligosaccharide structures so as to achieve a more uniform population of molecules. Or we may express the erythropoietin in a simpler cell type, such as Sf9 cells, which produces more primitive glycosylation. This system already exists and has been used by us in the past to produce recombinant human erythropoietin.

Our plans for the next four months include further structural studies to more carefully define the structural role of oligosaccharide in erythropoietin, to prepare sufficient erythropoietin dimers to carry out a pharmacokinetic study with them at the Naval Blood Research Laboratory and to begin to evaluate their efficacy in stimulating blood cell production in the rodent model.

2. **High Potency Erythropoietin.**

As noted previously, this work had been temporarily suspended because of unexplained variability in results. At a personal meeting with Capt. Carl June and Capt. Sheila Weinberg, on 7 June 1995 at Bethesda Naval Hospital, I was requested to provide greater detail regarding this unforeseen problem. Our difficulties can be summarized as follows. Derivitization of monomeric recombinant human erythropoietin with SPDP, LC-SPDP or sulfo-LC-SPDP results in a erythropoietin preparation with increased specific activity (U/mg) over the nonderivatized material. This enhancement has been found to range from 3-fold to more than 10-fold. In general, the enhancement is directly related to the molar ratio of SPDP to erythropoietin used in the reaction mixture up to 10/1. At higher molar ratios, this enhancement is reduced but not entirely eliminated. Unfortunately, from experiment to experiment, identical molar ratios of reactants do not yield identical degrees of enhancement. For example, in one experiment, a 10:1 ratio may yield 10-fold enhancement whereas in another experiment several days later the enhancement may only be 4-5 fold. The proof of concept remains unchallenged, namely, that derivatization of Epo with SPDP reagents results in a high potency compound. Rather the difficulty is with consistency of enhancement from preparation to preparation. Earlier in the course of this grant period, we had taken the position that such variability would make in vivo studies difficult to interpret. Moreover, we had no understanding of why this variation occurred among these individual preparations. We now believe that our structural studies, detailed above, which show a pronounced effect of oligosaccharides on Epo structure, provide at least a partial explanation for this variability. Structural differences among individual Epo molecules increase the variability in the system, making precise reproducibility from preparation to preparation extremely difficult.

It is still our intention to return to high-potency erythropoietin. However, because of our promising results with long-acting erythropoietin and because of the need to move forward in our collaboration with Dr. Valeri's laboratory, we chose to temporarily suspend this work and redeploy our personnel and supplies toward an increased effort and increased focus on long-acting erythropoietin. This budgetary redeployment is detailed below.

3. **Erythroid colony stimulating factor.**

Because of the reduction in budget ordered by the Office of Naval Research in the Spring of 1995, work on erythroid colony stimulating factor had been suspended. This was an agreed upon by Dr. Sytkowski and Capt. Sheila Weinberg previously. The redeployment of a small amount of funds remaining from this project to the long-acting Epo project is detailed below. The -03 year of this grant began on July 1, 1995. At that time, work on erythroid colony stimulating factor was started again. Work since that time has focused on two areas. The first is the study of E-CSF upregulation of the c-myb proto-oncogene in rodent erythroid cells. It is believed that upregulation of c-myb in hematopoietic cells coincides with a growth promoting stimulus, consistent with the action of E-CSF. However, since our other work on erythropoietin has demonstrated the downregulation of myb coincident with hemoglobin production, it is necessary for us to sort out the difference in the E-CSF signal and Epo signaling pathway in order to more fully understand the effects of E-CSF in the planned in vivo experiments. Secondly, we have recently begun to grow rodent bone marrow colonies in methyl cellulose semi-solid culture. This method, when fully developed, will have great advantage over the plasma clot assay described in a previous report. Cells can be harvested from methyl cellulose culture and studied further. In the next project period, work on E-CSF will continue to focus on its signaling to the c-myb protooncogene and on developing in vitro methyl cellulose culture techniques for all murine hematopoietic progenitors.

BUDGET - REDEPLOYMENT OF PERSONNEL AND SUPPLY FUNDS.

As discussed above, some redeployment of personnel and supply funds has been necessary in the course of this grant. Firstly, suspension work on high-potency erythropoietin caused us to redeploy all personnel and supply funds being used on that project to increase our effort on long-acting Epo. This consisted of approximately 10% of Dr. Sytkowski's effort, 25% of Dr. Lunn, 20% of research assistant Davis, and 10% of Mrs. Panza. Ten percent of the supply budget had been used for high potency Epo and was redeployed to long-acting Epo. Progress on long-acting Epo was helped significantly by this redeployment since the new problems that we had encountered due to structural differences among Epo molecules had slowed our progress somewhat. However, despite these new problems, we believe that our structural data gives us a means by which we can address them and overcome them. This still remains a project with some

risk, however the benefit to the Navy of a long-acting erythropoietin pharmaceutical greatly outweighs this risk. Moreover, the long-acting erythropoietin preparation will serve a dual use, being important to both the military and civilian sector. The second budgetary distribution took place in FY95 due to a budget reduction of $21,000. After conferring with Capt. Sheila Weinberg, our project officer, it was decided to suspend work on erythroid colony stimulating factor for the remainder of that fiscal year so as not to reduce the quality of science. Therefore, for the period beginning on the date of the budgetary reduction until the end of the second project year (June 30) all work on erythroid colony stimulating factor was suspended. The salary and personnel funds remaining in the budget for that year were transferred to long-acting Epo and resulted directly in our circular dichroism structural studies described above. This led to the major breakthrough that we made regarding the role of oligosaccharides in the polypeptide backbone folding. Since July 1, 1995, the third year of our grant, work on erythroid colony stimulating factor was reinstituted as described previously.

PRODUCTS

The following products not previously submitted have resulted from this grant. Copies of these papers accompany this report.

Romanowski RR, Sytkowski AJ. The Molecular Structure of Human Erythropoiein. In: Spivak JL, ed. Hematology/Oncology Clinics of North America: Erythropoietin: Basic and Clinical Aspects. Philadelphia: WB Saunders, 1994; 8:885-894.

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Keutzer JC, Sytkowski AJ. Regulated production of a pleiotropic cytokine - PDGF - by differentiating erythroid cells in vitro and in vivo. PNAS 1995; 92:4967-4971.

Patel HR, Sytkowski AJ. Erythropoietin activation of AP1 (fos/jun). Exp Hem. 1995;23:619-625.

Grodberg J, Davis KL, Sytkowski AJ. Functional and structural role of arginine 103 in human erythropoietin. Eur J Biochem; submitted.