EXHIBIT 6
Part 1 of 2

HL21676

Form approved OMB No. 0925-0001

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK		
TYPE	ACTIVITY	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL/BOARD (Month, year)		DATE RECEIVED

1. TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)
Erythropoietin: Purification, Properties, Biogenesis

2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT ☒ NO ☐ YES (If "YES," state RFA number and/or announcement title)

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
Goldwasser, Eugene

3b. SOCIAL SECURITY NUMBER
494-14-6535

3c. POSITION TITLE
Professor

3d. MAILING ADDRESS (Street, city, state, zip code)
The University of Chicago
920 East 58th Street
Chicago, Illinois 60637

3e. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Biochemistry & Molecular Biology

3f. MAJOR SUBDIVISION
Biological Sciences Division

3g. TELEPHONE (Area code, number and extension)
(312) 962-1348

4. HUMAN SUBJECTS
☐ NO ☒ YES ☐ Exemption # _____ OR ☒ Form HHS 596 enclosed

5. RECOMBINANT DNA
☐ NO ☒ YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD
From: 7/1/85 Through: 6/30/90

7. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD (from page 4)
$ 217,437

8. DIRECT COSTS REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD (from page 5)
$ 1,209,325

9. PERFORMANCE SITES (Organizations and addresses)
The University of Chicago
920 East 58th Street
Chicago, Illinois 60637

10. INVENTIONS (Competing continuation application only)
☒ NO ☐ YES ☐ Previously reported OR ☐ Not previously reported

11. APPLICANT ORGANIZATION (Name, address, and congressional district)
The University of Chicago
5801 South Ellis Avenue
Chicago, Illinois 60637
First Congressional District

Co. 13736

12. TYPE OF ORGANIZATION
☐ Public, Specify ☐ Federal ☐ State ☐ Local
☒ Private Nonprofit
☐ For Profit (General)
☐ For Profit (Small Business)

13. ENTITY IDENTIFICATION NUMBER
1362177139A1

14. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR BIOMEDICAL RESEARCH SUPPORT GRANT
Code [ ] Description School of Medicine

15. OFFICIAL IN BUSINESS OFFICE TO BE NOTIFIED IF AN AWARD IS MADE (Name, title, address and telephone number.)
Donald S. Sigal, Director, Office of Sponsored Programs
5801 S. Ellis Avenue
Chicago, Illinois 60637
312-962-8604

16. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Name, title, address and telephone number)
1)
2) Donald S. Sigal, Director, Office of Sponsored Programs (312) 962-8604

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001).
SIGNATURE OF PERSON NAMED IN 3a (In ink. "Per" signature not acceptable) Eugene Goldwasser
DATE 31 August 1984

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001).
SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)
DATE

PHS 398 (Rev. 5/82)

A 196380
CONFIDENTIAL

CONFIDENTIAL
SUBJECT TO COURT PROTECTIVE ORDER

TRIAL EXHIBIT
2057
97-10814 WGY

DEFENDANT'S DEPOSITION EXHIBIT
79
10-13-99

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Goldwasser, Eugene

Detach and clip this page (unnumbered) to the signed original of the face page of the application. Do not duplicate.

PERSONAL DATA ON PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

The Public Health Service has a continuing commitment to monitoring the operation of its review and award processes to detect—and deal appropriately with—any instances of real or apparent inequities with respect to age, sex, race, or ethnicity of the proposed principal investigator/program director.

To provide the PHS with the information it needs for this important task, the principal investigator/program director is requested to complete the form below and attach a single copy to the signed face page of the application.

Upon receipt and assignment of the application by the PHS, this form will be detached from the application. It will NOT be duplicated and will NOT be a part of the review process. Data will be confidential, and will be maintained in Privacy Act record system 09-25-0036, "Grants: IMPAC (Grant Contract Information)." All analyses conducted on the data will report aggregate statistical findings only and will not identify individuals.

If you decline to provide this information, it will in no way affect consideration of your application.

Your cooperation will be appreciated.

DATE OF BIRTH *(month/day/year)*	SEX
10/14/22	☐ Female ☒ Male

RACE AND/OR ETHNIC ORIGIN *(check one)*

- ☐ American Indian or Alaskan Native
- ☐ Asian or Pacific Islander
- ☐ Black, not of Hispanic origin
- ☐ Hispanic
- ☒ White, not of Hispanic origin

CONFIDENTIAL SUBJECT TO COURT PROTECTIVE ORDER

NOTE: The category that most closely reflects the individual's recognition in the community should be used for purposes of reporting mixed racial and/or ethnic origins. Definitions are as follows:

American Indian or Alaskan Native: A person having origins in any of the original peoples of North America, and who maintains cultural identification through tribal affiliation or community recognition.

Asian or Pacific Islander: A person having origins in any of the original peoples of the Far East, Southeast Asia, the Indian subcontinent, or the Pacific Islands. This area includes, for example, China, India, Japan, Korea, the Philippine Islands and Samoa.

Black, not of Hispanic origin: A person having origins in any of the black racial groups of Africa.

Hispanic: A person of Mexican, Puerto Rican, Cuban, Central or South American or other Spanish culture or origin, regardless of race.

White, not of Hispanic origin: A person having origins in any of the original peoples of Europe, North Africa, or the Middle East.

PHS 398 (Rev. 5/82)

A 196381
CONFIDENTIAL

Goldwasser , Eugene

ABSTRACT OF RESEARCH PLAN

NAME		
Eugene Goldwasser	Professor	Biochem. & Molec. Biol. The University of Chicago
Fung-Fang Wang	Research Associate(Instructor)	Biochem. & Molec. Biol. The University of Chicago
Phillip Maples	Post-doctoral trainee	Biochem. & Molec. Biol. The University of Chicago

CONFIDENTIAL
SUBJECT TO COURT PROTECTIVE ORDER.

DO NOT EXCEE

THE SPACE PROVIDED

The salient features of this proposal are: The study of those structural features of human erythropoietin that may be directly involved with its biological activity on hemopoietic precursor cells; the study of the primary structure of the active site; the determination of oligosaccharide side chain structure; the isolation and characterization of specific cellular receptors for erythropoietin from cells infected with the anemia strain of the Friend virus; the extension of receptor studies to normal mouse cells and to apply the methods developed for that purpose to the study of human cellular receptors. Once isolated the erythropoietin receptor will be cloned and the gene for the receptor used to test an hypothesis regarding erythroid cell differentiation. Mouse erythropoietin will also be cloned and the DNA used for the study of the regulation of expression of the erythropoietin gene as well as for the study of erythropoietin biogenesis and secretion. Another aim is to develop a solid-phase radioimmunoassay using monoclonal anti-epo with the intention of improving the sensitivity of analysis by an order of magnitude. The same monoclonal anti-epo will be used as the basis for an improved immunoaffinity purification method.

81-3741

VERTEBRATE ANIMALS INVOLVED XX

mice, rats

PAGE 2

A 196382
CONFIDENTIAL

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Goldwasser, Eugene

TABLE OF CONTENTS

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator/Program Director at the top of each printed page and each continuation page.

Number of publications: 16 Number of manuscripts: 6

Other items *(list)*:

Letter from Dr. Krantz
Letter from Dr. Rowley
Table I
Figure 1
Figure 2
Distribution sheets

☒ Application Receipt Record, Form PHS 3830
☒ Form HHS 596 if Item 4, page 1, is checked "YES" and no exemptions are designated.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Goldwasser, Eugene

DETAILED BUDGET FOR FIRST 12 MONTH BUDGET PERIOD
DIRECT COSTS ONLY

FROM 7/1/85 THROUGH 6/30/86

DOLLAR AMOUNT REQUESTED (Omit cents)

PERSONNEL (Applicant organization only) NAME	POSITION TITLE	TIME/EFFORT %	TIME/EFFORT Hours per Week	SALARY	FRINGE BENEFITS	TOTALS
Eugene Goldwasser	Principal Investigator	20		13,090	2,605	15,695
Fung-Fang Wang	Res. Assoc (Instr)	100		23,881	4,752	28,633
Charles K.-H. Kung	Res. Technologist		40	31,686	6,464	38,150
Annette Gardner	Res. Technician		40	23,026	4,697	27,723
Carol Sims	Res. Technician		40	18,589	3,792	22,381
Catherine Fowler	Lab Assistant		40	17,607	3,592	21,199
Yvonne Price	Secretary		20	9,459	1,930	11,389
Anthony Kittler	Instrument Designer		2.5	2,053	419	2,472
	SUBTOTALS →			139,391	28,251	167,642

CONSULTANT COSTS

EQUIPMENT (Itemize)
Rotary Incubator — 3,500

SUPPLIES (Itemize by category)
200 rats @ $5.82 ea. = $1,164; 300 mice @ $3.75 ea. = $1,125; 100 nude mice @ $60 ea = $6,000; isotopes $3,945; chemicals $6,480; media $5,000; glass and plastic ware $4,000; HPLC columns $6,000; film $1,000; enzymes $1,656; — 36,370

TRAVEL	DOMESTIC one relevant trip for PI and Res. Assoc.	2,000
	FOREIGN	0
PATIENT CARE COSTS	INPATIENT	0
	OUTPATIENT	0

ALTERATIONS AND RENOVATIONS (Itemize by category) — 0

CONSORTIUM/CONTRACTUAL COSTS — 0

OTHER EXPENSES (Itemize by category) animal care $960; (4,000 mouse days @ 0.12 = $480; 2,000 rat days @ 0.24=$480); maintenance contracts, $3,750; radiation protection, $1,250; long distance phone, $200; book and journals $100; publication costs, $1,300; (page charges $500; reprints $800); copying $200; postage $165. — 7,925

TOTAL DIRECT COSTS (Also enter on page 1, item 7) → $217,437

PHS 398 (Rev. 5/82) PAGE 4

A 196384
CONFIDENTIAL

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Goldwasser, Eugene

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits.) (Applicant organization only)		167,642	177,701	188,363	199,665	211,645
CONSULTANT COSTS		-	-	-	-	-
EQUIPMENT		3,500	5,000	0	0	0
SUPPLIES		36,370	38,189	40,098	42,103	44,208
TRAVEL	DOMESTIC	2,000	2,100	2, 205	2,315	2,431
	FOREIGN					
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
CONSORTIUM/ CONTRACTUAL COSTS						
OTHER EXPENSES		7,925	8,321	8,737	9,174	9;633
TOTAL DIRECT COSTS		217,437	231,311	239,403	253,257	267,917

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Also enter on page 1, item 8) → $ 1,209,325

JUSTIFICATION (Use continuation pages if necessary): Describe the specific functions of the personnel and consultants. If a recurring annual increase in personnel costs is anticipated, give the percentage. For *all* years, justify any costs for which the need may not be obvious, such as equipment, foreign travel, alterations and renovations, and consortium/contractual costs. For any additional years of support requested, justify any significant increases in any category over the first 12 month budget period. In addition, for COMPETING CONTINUATION applications, justify any significant increases over the current level of support.

"The inclusion of faculty salary in this budget is a policy of the Division of the Biological Sciences and The Pritzker School of Medicine. If an award is made in a reduced amount, the Principal Investigator will be asked to retain an appropriate amount of faculty salary support in the budget.

Most appointments in the Division of the Biological Sciences and The Pritzker School of Medicine are on a full-time, 12 month basis. Although salaries are assured on this basis, it is expected that investigators will recover reasonable and appropriate salary support from research grants and contracts proportionate to the fraction of their time and effort devoted to the research."

8:63744

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR OR AWARD CANDIDATE (Last, first, middle)	SOCIAL SECURITY NUMBER
Goldwasser, Eugene	494-14-6535

Personnel: The budget for the first period is 11.1% greater than the current budget due to increased effort of the P.I. and to mandated increases in non-academic salaries. The percent effort of the P.I. has been increased from 15 to 20, a desirable increase, but reflected in the increased budget. Dr. Wang is a full-time Research Associate (Instructor) who is responsible for structural studies of both epo and epo-receptors. She will also work on receptor cloning as results permit. Mr. Kung is the pivotal person in this lab; as the chief Research Technologist he is responsible for purification procedures, studies of labeling techniques, analysis and preparation by HPLC and immunoaffinity methods. He also will be responsible for blotting techniques (Southern, Northern and Western). Ms. Gardner is responsible for routine RIA and work on new RIA methods, as well as assay result bookkeeping. Ms. Sims is responsible for marrow culture assays, for some work on purification and for maintenance of cell lines. Ms. Fowler is a lab assistant who is responsible for dishwashing, sterilization, lab orderliness and copying. Ms. Price, as secretary takes care of ordering, logging in received orders, typing of manuscripts and reports, and some bookkeeping. Mr. Kittler is needed for minor repair work, instrument design and construction. Some of the work to be done will be carried out by graduate students and post-doctoral fellows who are not, at this time, carried as personnel on this project.

Equipment: In the first period we request funds for a rotary incubator to be used to grow bacterial cells on DNA cloning. At present we have to borrow the use of one already heavily used on another floor. We need this incubator for growing cells with plasmids for cloning the mouse epo gene and for the receptor gene. In the second period we request funds for a low temperature freezer; at present we share (1/3) one and the capacity is clearly too small for our needs. Many of our RNA and DNA supplies require ultra low temperature storage and this type of freezer is required.

Other expenses: We have a sizable budget item for maintenance contracts; this includes partial costs of maintaining the following: two gamma counters, a liquid scintillation spectrometer, three Sorvall centrifuges, a Beckman L5-65 and an autoclave. We are convinced that there is a saving involved since everyone of these is essential and we would have to pay premium prices to get repairs without such contracts.

Personnel costs have been projected to increase at 6% per year, all other costs at 5% per year. Except as indicated above, and for the equipment requested, the budgetary increment over the present grant is about 5%.

DO NOT TYPE IN THIS SPACE—BINDING MARGIN

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR. Goldwasser, Eugene

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Eugene Goldwasser	Professor of Biochemistry	10/14/22

EDUCATION (Begin with baccalaureate or other initial professional education and include postdoctoral training)

INSTITUTION AND LOCATION	DEGREE (circle highest degree)	YEAR CONFERRED	FIELD OF STUDY
The University of Chicago, Chicago, Il.	S.B.	1943	Biochemistry
The University of Chicago, Chicago, Il.	Ph.D.	1950	Biochemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Positions:

Research Associate: Department of Biochemistry, The University of Chicago 1952-1961
Associate Professor of Biochemistry: The University of Chicago 1962-1963
Professor of Biochemistry, The University of Chicago 1963-present
Chairman, Committee on Developmental Biology, The University of Chicago 1976-present
Chairman, Dept of Biochemistry & Molecular Biology 1984-present

Honors:

Guggenheim Fellowship Oxford University, U.K. 1966-1967
AAAS Fellow

Publications:

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

Weiss TL and Goldwasser E. The biological properties of endotoxin-free human erythropoietin. Biochem. J 98:17-21, 1981.

Goldwasser E. Erythropoietin and red cell differentiation in Control of Cell Division and Development. Eds. D Cunningham, E Goldwasser, D Watson and CF Fox pp 487-494, AR Liss, New York, 1981.

Goldwasser E and Sherwood JB. Radioimmunoassay of erythropoietin. Brit. J. Haematol. 98:359-364, 1981.

Tong BD and Goldwasser E. The formation of erythrocyte membrane proteins during induced differentiation. J Biol. Chem. 256:19222-12672, 1981.

Distelhorst CS, Wagner DS, Goldwasser E and Adamson JW. Autosomal dominant familiar erythrocytosis due to autonomous erythropoietin production. Blood 56:1155-1158, 1981.

Ely JM, Prystowsky MB, Eisenberg L, Quintans J, Goldwasser E, Glasebrooke AL and Fitch FW. Alloreactive cloned T cell lines. J. Immun. 127:2345-2349, 1981.

Nijhof W, Wiergena PK and Goldwasser E. The regeneration of stem cells after a bone marrow deparession induced by thiamphenicol. Exp. Hematol. 10:36-43, 1982.

Goldwasser E. Some thoughts on the nature of erythropoietin-responsive cells. J. Cell. Physiol. Suppl. 1. pp 133-137, 1982.

Weiss TL, Kavinsky C and Goldwasser E. Characterization of a monoclonal antibody to human erythropoietin. Proc. Natl. Acas. Sci. 79:5465-5469, 1982.

Shalhoub RM, Rajan U, Kim VV, Goldwasser E, Kark JA and Antoniou LD. Erythrocytosis in patients on long-term hemodialysis. Ann. Int. Med. 97:686-690, 1982.

Prystowsky MD, Ely JM, Beller DI, Eisenberg L, Goldman J, Goldman M, Goldwaaser E, Ihle J, Quintans J, Remold H, Vogel SN and Fitch FW. Alloreactive cloned T cell lines VI. Multiple lymphokine activities secreted by helper and cytolytic cloned T lymphocites. J. Immunol. 129:2337-2344, 1982.

Kawakita M, Ogawa M, Goldwasser E and Miyake T. Characterization of human megakaryocyte colony-stimulating factor in the urinary extracts from patients with aplastic anemia and idiopathic thrombocytopenic purpura. Blood, 61:556-560, 1983.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR OR AWARD CANDIDATE (Last, first, middle)	SOCIAL SECURITY NUMBER
Goldwasser, Eugene	494-14-6535

Goldwasser E. Editor, Regulation of hemoglobin synthesis: The Third Symposium of The University of Chicago Comprehensive Sickle Cell Center. Elsevier, New York, 1983.

Prystowsky MB, Ely J, Vogel SN, Goldwasser E and Fitch FW. Biochemical enrichment of lymphokines secreted by a cloned helper T lymphocyte. Fed Proc. 42:2757-2761, 1983.

Beru N, Sahr K and Goldwasser E. Inhibition of heme synthesis by succinylacetone: Effect on globin synthesis in bone marrow cells. J. Cell. Biochem. 21:93-105, 1983.

Sahr K and Goldwasser E. The effect of erythropoietin on the biosynthesis of translatable globin mRNA. in Regulation of hemoglobin biosynthesis. Ed. E. Goldwasser, Elsevier, New York, p 153-161, 1983.

Lappin TRJ, Rich I and Goldwasser E. The effect of erythropoietin and other factors on DNA synthesis by mouse spleen cells. Exp. Hematol. 11:661-666, 1983.

Wang FF and Goldwasser E. The purification of a human urinary colony-stimulating factor. J. Cell Biochem. 21:263-276, 1983.

Goldwasser E, Ihle JN, Prystowsky MD, Rich I and Van Zant G. The effect of interleukin-3 on hemopoietic precursor cells. in Symposium on Normal and Neoplastic Hematopoiesis, eds. DW Golde, and PA Marks, AR Liss, p. 301-310, 1983.

Weiss TL, Kung CKH and Goldwasser E. Erythropoietin binding to bone marrow and spleen cells. in Symposium on Normal and Neoplastic Hematopoiesis, eds. DW Golde and PA Marsk, AR Liss, p 455-464, 1983.

Prystowsky MB, Ihle JN, Otten G, Keller J, Rich I, Naujokas M, Loken M, Goldwasser E and Fitch FW. Two biological distinct colony-stimulating factors are secreted by a T lymphocyte clone. in Symposium on Normal and Neoplastic Hematopoiesis. eds. DW Golde and PA Marks, AR Liss, p. 369-378, 1983.

Prystowsky MB, Ely JM, Naujokas MF, Goldwasser E and Fitch FW. Partial purification and characterization of a colony-stimulating factor secreted by a T-lymphocyte clone. Exp. Hematol. 11:931-143, 1983.

Prystowsky MB, Naujokas MF, Ihle JN, Goldwasser E and Fitch FW. A Microassay for colony-stimulating factor based on thymidine incorporation. Amer. J. Path. 114:149-156, 1984.

Hopfer SM, Sunderman FW, Reid MC and Godlwasser E. Increased immunoreactive erythropoietin in serum and kidney extracts of rats with NI_3S_2 induced erythrocytosis. Res. Commun. Chem. Path. Pharm. 43:299-305, 1984.

Van Zant G and Goldwasser E. Erythropoietin and its target cells. in Growth and Maturation Factors, ed. G Guroff, John Wiley, New York, 1984.

Emmanouel DS, Goldwasser E and Katz, AI. Metabolism of pure human erythropoietin in the rat. Am. J. Physiol. 247:168-176.

Krantz SB and Goldwasser E. Specific binding of erythropoietin to spleen cells infected with the anemia strain of Friend virus. Proc. Nat. Acad. Sci. in press. 1984

Goldwasser E. The characteristics and function of factors affecting erythropoiesis. Kroc Foundation Symposium, in press, 1984.

Goldwasser E, Krantz SB and Wang FF. Erythropoietin and erythroid differentiation MD Anderson Symposium, in press, 1984.

Weiss TL, Kung CKH and Goldwasser E. The frequency of bone marrow cells that bind erythropoietin. J. Cell Biochem. in press, 1984.

Sherwood JB and Goldwasser E. Erythropoietin production by human renal carcinoma cells in culture. Endocrinology, 99:504-510, 1976.

Miyake T, Kung CKH and Goldwasser E. Purification of human erythropoietin. J. Biol. Chem. 252:5558-5564, 1977.

Sherwood JB and Goldwasser E. Extraction of erythropoietin from normal kidneys, Endocrinol. 103:866-870, 1978.

Eliason JF, Van Zant G and Goldwasser E. The relationship of hemoglobin synthesi to erythroid colony and burst formation. Blood, 53:935-946, 1979.

Van Zant G and Goldwasser E. Competition between erythropoietin and colony-stimu lating factor for target cells in mouse marrow. Blood, 53:946-965, 1979.

Sherwood JB and Goldwasser E. A radioimmunoassay of erythropoietin. Blood 54:885-893, 1979.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR. Goldwasser, Eugene

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Fung-Fang Wang	Research Associate	5/5/48

EDUCATION (Begin with baccalaureate or other initial professional education and include postdoctoral training)

INSTITUTION AND LOCATION	DEGREE (circle highest degree)	YEAR CONFERRED	FIELD OF STUDY
National Taiwan Univ. (Taipei, Taiwan)	B.S.	1970	Agricultural chemist.
Rutgers Univ. (New Brunswick, NJ)			Biochemistry
Indiana Univ. (Bloomington, IN.)	Ph.D.	1977	Chemistry
City of Hope Med Ctr(Duarte,CA.)			Immunology
Univ. of Chicago. (Chicago, Il.)			Biochemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

1977-1978 City of Hope Medical Center Junior Research Scientist
Purification and characterization of fibronectin and carcino embryonic antigen.

1979-present The Univ. of Chicago Research Associate
Purification and characterization of human urinary colony stimulation factor, structure studies of erythropoietin. Binding of epo to its receptor, Partial purification and characterization of a colony stumulating factor from our embryonic kidney cell line.

PUBLICATIONS:

1. F. F. Wang and E. Goldwasser. 1983 Purification of a human urinary colony stimulating factor. J. Cell. Biochem. 21:263-275.
2. F.F. Wang and E. Goldwasser. 1983 Some chemical properties of erythropoietin. Fed. Proc. 42:1872(abstract).
3. F.K. Lin, C.H. Lin, S. Suggs, P.H. Lai, R. Smalling, J. Browne, J. Egrie, F.F. Wang and E. Goldwasser. 1984, Cloning and expression of the monkey erythropoietin gene. Fed. Proc. 43:1724.
4. F.F. Wang, C.K.H. Kung and E. Goldwasser. Some chemical properties of human erythropoietin. (submitted to Endocrinology for publication).
5. M.S. Dordal, F.F. Wang and E. Goldwasser. The role of carbohydrate in erythropoietin action. (Submitted to Endocrinology for publication).
6. E. Goldwasser, S.B. Krantz and F. F. Wang. 1984. Erythropoietin and erythroid differentiation. M.D. Anderson Symposium (in press).

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR Goldwasser, Eugene

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Phillip B. Maples	Post-doctoral trainee	6/27/56

EDUCATION (Begin with baccalaureate or other initial professional education and include postdoctoral training)

INSTITUTION AND LOCATION	DEGREE (circle highest degree)	YEAR CONFERRED	FIELD OF STUDY
University of Tulsa,	B.S.	1978	Microbiology
Graduate Coll of Med.,Oklahoma Univ Health Sciences Center	Ph.D.	1984	Biochem. & Molec. Biol

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

8/1976-6/1978 Medical Technician, Hillcrest Medical Center, Tusla Oklahoma.
Jan-May, 1978 Lab Assistant, Dept of Biology, Univ of Tulsa, Tulsa, Ok.
Jan-May, 1978 Lab Assistant, Dept of Chemistry, Univ of Tulsa, Tulsa, OK.
11,1978-6,1979. Research Technician, Dept of Biochem. & Molec. Biology, OU Health Sciences Center, Oklahoma City, OK.
7,1979-6,1982. Graduate Res. Assistant, Dept of Biochemistry and Molecular Biology OU Health Sciences Center, Oklahoma City, OK.

PUBLICATIONS

1. Broyles, R.H., G.M. Johnson, P.B. Maples and G.R. Kindell. Two erythropoietic microenvironments and two cell lines in bullfrog tad-.poles. Devel. Biol. 81: 299-314, 1981.

2. Broyles, R.H., A.R. Dorn, P.B. Maples, G.M. Johnson, G.R. Kindell and A.M. Parkinson. Choice of hemoglobin type in erythroid cells of Rana catesbeiana. in Hemoglobin in Development and Differentiation (B. Stamatoyannopoulos and A.W. Neinhuis, eds.), Alan R. Liss, Inc., New York, 1981.

3. A.M. Parkinson, A.R. Dorn, P.B. Maples and R. H. Broyles. Improved electrophoretic separation of hemoglobins by standard PAGE with different amino acid buffers. Anal. Biochem. 117:6-11, 1981.

4. P.B. Maples, A.R. Dorn and R.H. Broyles. Coexistance of embryonic and larval hemoglobins during the early development of the bullfrog Rana catesbeiana. Devel. Biol. 96:515-519, 1983.

5.

08 C3749

CONFIDENTIAL
SUBJECT TO COURT PROTECTIVE ORDER

A 196390
CONFIDENTIAL

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Goldwasser, Eugene

OTHER SUPPORT

(Use continuation pages if necessary)

For each of the professionals named on page 2, list, in three separate groups: (1) active support; (2) applications and proposals pending review or funding; (3) applications and proposals planned or being prepared for submission. Include *all* Federal, non-Federal, and institutional grant and contract support. If none, state "none." For each item give the source of support, identifying number, project title, name of principal investigator/program director, time or percent of effort on the project by professional named, annual direct costs, and entire period of support. (If part of a larger project, provide the titles of both the parent project and the subproject and give the annual direct costs for each.) Describe the contents of each item listed. If any of these overlap, duplicate, or are being replaced or supplemented by the present application, delineate and justify the nature and extent of the scientific and budgetary overlaps or boundaries.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR:

(1) ACTIVE SUPPORT:

NIH Grant CA 18375; Hemopoietic Stem Cells and Induced Differentiation, P.I. Eugene Goldwasser (20%), direct costs 05/01/84 to 04/30/85, $80,364, period of support 07/01/78 to 06/30/88.

NIH Grant HL 30121; Program Project, The Biology of Sickle Cell Disease, P.I. Eugene Goldwasser (10%), 04/10/84 to 03/31/85; Sub Project VI, Study of the Regulation of Hemoglobin Synthesis in Bone Marrow Cell. Direct costs $57,406, period of support 04/01/83 to 03/31/88.

2. Pending
This application - 09 yr HL 21676

3. Planned
None

C8:23750

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Goldwasser, Eugene

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used at the applicant organization and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe the facilities at any other performance sites listed in Item B, page 1, and at sites for field studies. Using continuation pages if necessary, include an explanation of any consortium arrangements with other organizations.

[X] Laboratory: Approx. 1500 sq. ft: fully operating, including culture labs, and needed equipment, and a cold room.

[XX] Clinical: When needed, the Clinical Research Center can be made available. It can be used for any further clinical testing.

[XXX] Animal: Carlson Animal Research Facility is used to house all lab animals and to maintain them in a healthy state for experimental purposes.

[XXX] Computer: A micro computer with hard copy and graphics output.

[XXX] Office: There are separate offices for the P.I. and the secretary.

[ ] Other (_____):

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

HPLC, Gas chromatograph, culture hoods, incubators, centrifuges, monitors β and γ counters, spectrophotometers are all within the lab.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Secretarial service within the lab; the machine shop is an important adjunct as noted in the budget justification.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR OR AWARD CANDIDATE (Last, first, middle)	SOCIAL SECURITY NUMBER
Goldwasser, Eugene	494-14-6535

SPECIFIC AIMS

Among the various polypeptide cellular growth factors that have been under study in recent years, erythropoietin (epo) occupies a special position. Its existence has, probably, been known longer than any other growth factor, yet much less is known about its chemistry and mode of action. This is due, clearly, to the very limited quantities of pure epo available. Another special aspect of epo biology relates to its high degree of specificity; unlike many of the other growth factors, the result of epo action is the formation of a single class of differentiated blood cells, erythrocytes.

We propose, here, to continue our intensive study of both the chemistry and biology of epo and to continue, as well, the extension of our laboratory work to possible clinical applications. More specifically, we plan to devote considerable effort to the study of the structure of epo in order to understand the chemical basis of its specific biological activity. We especially want to study the structure of the active region (or regions) and the relationship between that structure and its interaction with specific cellular receptors of epo. Simultaneous with these studies we plan to continue to work on the possibility that smaller and simpler fragments of epo may be biologically active. Both of these kinds of study, necessarily will involve further investigation of the epo receptor and we plan to extend our work in this field in two directions: the further chemical characterization of the receptor from virus-infected cells and the study of normal cell receptors.

We plan to use our newly developed tools, such as the monoclonal anti-epo and the cloned epo DNA, to study the regulation of epo biogenesis in normal kidney and/or fetal liver cells. In addition, the current availability of a line of mouse cells that constitutively secretes substantial amounts of epo into the medium, will make it possible to study the path of biosynthesis of this glycoprotein.

We plan to also extend our studies of epo levels in disease states, but will first concentrate on improving the current RIA by using the monoclonal anti-epo, developed in this lab, in solid state assay which will rely on the antibody for specificity rather than on pure epo.

Lastly, we will continue our several collaborations with other laboratories in the study of both clinical and experimental aspects of epo biology.

SIGNIFICANCE

The central role of epo in the normal regulation of mammalian red cell formation is now well established, as well as its importance in a wide array of fields such as, clinical hematology, experimental hematology, cell differentiation, hormone action and eukaryotic gene expression. Study of the biochemistry and molecular biology of epo and of its molecular and cellular modes of action are now more timely than ever.

These are several aspects of the research planned in this proposal; some of these are now under study in this laboratory and we propose to continue with them. The subjects we are now investigating or plan to study and the rationale for each follow:

Purification Until the mass production of biologically active epo, based on recombinant DNA is accomplished, there will be a real need for "natural" epo from urine, plasma or culture media. Since all of these sources are limited, improved purification methods will be needed; improved especially with respect to yield. We proprose to continue our work[1] on developing a rapid, simple and high yielding method which will, in addition, be applicable to epo produced by recombinant methods as well.

Structure Because of the striking specificity of epo action in the induction of red cell formation, it is of general importance with respect to cell differentiation to understand the detailed mechanism of how this particular glycoprotein exerts its effect. Ultimately the action of epo on its target cells must be a function of its structure and the structure of its receptor. There are several aspects to the structure of a glycoprotein that should be studied: the primary structure of the polypeptide. the primary structures of the oligosaccharide chains and the secondary and tertiary structures of the holoprotein. Our working hypothesis is that there is a region of the polypeptide that is less tightly structured than the remainder and that interacts

with a specific epo receptor on responsive cells. The conformation of this active region may be dependent, in part, on the secondary and tertiary structures which may be defined by the relationship between the hydrophilic oligosaccharides and the generally hydrophobic protein.

We have shown that immunoreactive material, smaller in molecular size than native epo, (termed "fragments" for convenience) can be found in the sera of some patients with chronic renal disease.[2] We propose to isolate and characterize these fragments with the idea that one or more may contain the active region of epo and may interact with receptors on hemopoietic precursor cells and thus block epo action. If this proves to be the case we may find an explanation for some of the anemias of chronic renal disease and perhaps other anemias. In addition, there remains the possibility that a fragment smaller than native epo may be biologically active.

Cloning of epo Now that the human epo gene has been cloned[3,4] and work is underway to produce epo commercially in large quantity, we plan to use similar methods to obtain cloned epo DNA from mouse, rat and rabbit to compare sequences and to study common structural features that may be important in biological activity. Probes derived from this area of research will be useful in study of biogenesis as outlined below.

Biosynthesis of epo The isolation of IW32 cells (a mouse line)[5] that make substantial quantities of epo in culture now makes it possible to study the path of its biosynthesis. We plan to examine the questions of whether epo is produced as a larger precursor, whether there are regions of the putative precursor that are required for transmembrane passage leading to secretion and what mechanisms regulate glycosylation of the protein. Since the secretion of up to 1U/ml appears to be constitutive, we plan to determine whether increased secretion by these cells can be affected by addition of substances known to have an effect in vivo. If so, we will be able, then, to study the mechanism of regulation of epo secretion and/or biosynthesis. This problem has not been able to be studied rigorously in the past. The question of how expression of the epo gene in these cells, and others, is regulated will also be studied using a specific nucleotide probe capable of hybridizing with epo mRNA.

Radioimmunoassay Investigation of many problems in epo biochemistry and physiology requires a rapid, specific and highly sensitive assay method. None of the assay systems available at present meets all of these requirements. We propose to study the development of a solid-state immunoassay, based on the monoclonal anti-epo developed in this laboratory with the requisite sensitivity and speed of analysis.

Epo receptor studies Now that we have shown the existence of specific receptors for epo in Friend cells (anemic variant, FVA)[6], we plan to extend this work in two directions, the purification and characterization of the FVA mouse receptors and the extension of receptor studies to normal erythropoietic cells. These problems are closely connected to our need to know the mechanism by which epo exerts its effect on target cells. One key problem in the study of cell differentiation, in general, and of red cell formation in particular, lies in the interaction between inducer (ligand) and sensitive cell. The question of whether the cellular program, resulting in massive hemoglobin synthesis, is set in motion by internalized epo-receptor complexes or whether by a trans-membrane signal, not involving internalization, cannot be answered without detailed knowledge of the specific receptor and its properties.

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

Goldwasser, Eugene | 494-14-6535

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 aggluinin (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 ^3H 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 ^{3}H 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

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.

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.

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.

PRINCIPAL INVESTIGATOR, PROGRAM DIRECTOR OR AWARD CANDIDATE (Last, first, middle)	SOCIAL SECURITY NUMBER
Goldwasser, Eugene	494-14-6535

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.

---

*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.

DO NOT TYPE IN THIS SPACE—BINDING MARGIN

08603761

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}C 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