EXHIBIT 2

Anlage B12
Nichtigkeitsklage ./. DE 34 82 828
Aktenzeichen 3 Ni 34/99 (EU)
F. Hoffmann-La Roche ./. Kirin-Amgen, Inc.
Uexküll & Stolberg
Beselerstraße 4, 22607 Hamburg

DECLARATION OF STUART H. ORKIN, M.D.

I, Stuart H. Orkin, do hereby declare as follows:

1. I make this declaration in support of EP 148 605 to Kirin-Amgen, Inc. entitled "Production of Erythropoietin." I have reviewed this patent and am familiar with its contents.

2. I currently hold the position of Leland Fikes Professor of Pediatric Medicine at Harvard Medical School in Boston, Massachusetts, USA. I have been on the faculty at Harvard Medical School since 1978. Attached as Exhibit A is my curriculum vitae which describes my educational and professional qualifications, awards and honors received, membership in professional societies and positions on editorial boards of professional journals. I am a member of both the National Academy of Sciences and the American Academy of Arts and Sciences. For the past twenty years, I have been involved in research in the area of molecular genetics and hematology. As shown by my curriculum vitae, I have authored or co-authored over 200 publications in these and related areas. For several years, I worked on research related to erythropoietin, and as part of that work, I had a research project in which I attempted to clone the gene that encodes human erythropoietin ("EPO gene"). Accordingly, I have first-hand knowledge of the difficulties of this project.

3. I have read the declaration of Arthur J. Sytkowski dated May 28, 1993 and submitted on behalf of Elanex Pharmaceuticals in these proceedings. I am the same Stuart H. Orkin mentioned in paragraphs 6 through 11 of Dr. Sytkowski's declaration, and I am submitting this declaration to respond to some of the statements made therein concerning my research efforts to clone the EPO gene. In sum, since I tried for over two years and was unsuccessful in cloning the EPO gene, I do not agree with Dr. Sytkowski's argument that as of 1983 it was expected that "the human gene coding for

erythropoietin would be cloned and that recombinant DNA technology would be the route of choice for erythropoietin production." If the cloning of the EPO gene was really as easy or expected as Dr. Sytkowski suggests, we should have been able to accomplish it. The facts are that it was a very difficult project, and I believe that my efforts exemplified the practical difficulties encountered in attempting to clone the EPO gene in 1983.

4. As described by Dr. Sytkowski in his declaration, I was the project leader on a research project entitled "Molecular Aspects of Globin Gene Switching and Cloning of Erythropoietin Sequence" which was funded under a grant to Dr. David G. Nathan from the National Institute of Health. As the title indicates, this project involved two major research efforts: (1) an investigation into the expression levels of the two alpha globin genes, and (2) cloning the cDNA sequences for erythropoietin. The proposals for this project are outlined on pages 8-10 of the draft Project Site Visit Report attached to Dr. Sytkowski's declaration. The project was originally designated for funding for five years, beginning in 1981.

5. We were successful in accomplishing the work relating to expression levels of the alpha globin genes within the first few months of beginning our study, and we published the results in late 1981. I believe this is an example of the level of competency of our research. As for the EPO project, we worked on this for over two years, and by the end of 1983, we had tried several approaches all of which proved unsuccessful. Since we had encountered obstacles that we could not overcome, the project was essentially abandoned.

6. Attached hereto as Exhibits B and C are the relevant sections of the yearly progress reports for the first two years of the EPO gene cloning project, 1981-82 and 1982-83, respectively. These reports document the work conducted during those years and the progress made toward achieving the goals established for those years. During the first year of the study, as indicated on pages 10 and 11 of Exhibit B, we pursued two approaches to cloning the DNA sequence that encodes

EPO. As a prelude to both approaches, we looked for a tissue sample most likely to provide a suitable source for EPO mRNA. By keeping baboons under severe chronic hypoxemic stress (or anemic conditions) to augment EPO production, we isolated tissue and serum samples having EPO levels more than a 1000 times normal levels. We thus believed that hypoxic baboon kidney tissue was a good source of enriched message for EPO.

7. We then prepared undegraded mRNA from the baboon kidney tissue which was translated in vitro and we attempted to immunoprecipitate EPO protein using antibodies provided to us by Dr. Joseph Garcia who had developed EPO antibodies at Lawrence Laboratories in Berkeley, California. However, these immunoprecipitation experiments failed because the antibodies precipitated numerous other proteins produced in the baboon kidneys. Samples of translation mixtures which we believed contained mRNA for EPO were subjected to radioimmunoassay by Dr. Garcia but no EPO could be detected above background. Thus, the lack of specificity of the antibody preparation precluded identification of translated EPO mRNA. One of the difficulties of the EPO project was that naturally occurring EPO is produced in very small quantities by unknown cells in the kidney, thus classifying it as a "rare" protein. Since there is only a low level of protein produced, this means that only a small amount of mRNA is present in the source tissue. This difficulty was in contrast to many of the other more abundant proteins for which genes were cloned in the early 1980's, e.g., alpha interferons, because the mRNA for those proteins was present in more abundant and detectable quantities.

8. During the second year of the study, as indicated on page 9 of Exhibit C, we continued our efforts to clone the EPO gene sequences. We prepared cDNA libraries from the hypoxic baboon kidney mRNA described above and also from human fetal liver which was believed to be an enriched source of EPO-mRNA. These cDNA libraries were then screened with mixtures of probes which were designed based on the N-terminal amino acid sequence of EPO as reported by

Goldwasser. This was the same sequence published by Dr. Sytkowski in his 1983 Sue et al. publication.

9. In our initial cloning attempts, we employed two pools of fully degenerate 17-mer oligonucleotide probes that corresponded to the N-terminal amino acids 21-26 (pool 1) and 18-23 (pool 2) from the Goldwasser sequence. Clones from the baboon kidney library were isolated that specifically hybridized to each of the pools under maximum stringency. Selected plasmid clones were then subjected to DNA sequencing to identify the region that hybridized with the oligonucleotide probes. After screening more than 250,000 independent baboon cDNA clones with both sets of probes, we obtained more than two dozen putative EPO clones that were then separated into classes by cross-hybridization of inserts. DNA sequencing revealed that we had, in fact, isolated clones with perfect matches for the oligonucleotide probes employed, however, the sequences were incompatible with the surrounding N-terminal EPO protein sequence. No clones hybridized with both oligonucleotide pools 1 and 2. We interpreted this to mean that either no clones contained EPO sequences or that the peptide sequences used for synthesis of one (or both) oligonucleotide pools were incorrect. Similar attempts to identify EPO DNA by screening the fetal liver cDNA library gave similar negative results.

10. We then used oligonucleotides directed to amino acids 6-11 (pool 3) and 11-16 (pool 4) of the Goldwasser sequence. Because these oligonucleotide pools were considerably more degenerate (greater than 512 individual sequences) we elected to determine whether any clones that hybridized with pools 3 or 4 also hybridized specifically with pools 1 or 2, based on the logic that a clone that hybridized to both degenerate pools would likely contain true EPO sequences. However, no such double-hybridizing clones were observed. Further, when Dr. Sytkowski had isolated his initial N-terminal peptide antibody, we examined its potential for antibody screening of the plasmid library. In preliminary experiments, we decided that the affinity of the antibody was probably insufficient for detection of bacterial products.

11. In designing our study, we adopted a cDNA cloning approach because that seemed to be the most workable methodology based on what was known in the art. Our experience, however, shows the practical difficulties commonly encountered in 1983 in gene cloning experiments. These difficulties were compounded for the EPO project because so little was known about EPO. The amino acid sequence information which we used to design our oligonucleotide probes proved to be unreliable. In addition, concerning the baboon cDNA screening, nothing was known concerning baboon EPO, and we did not know the similarity of the baboon EPO sequence to the human EPO amino acid sequence. Another difficulty was in identifying a good source for cDNA cloning because the specific EPO-producing cell type within the liver or kidney was not known. Also, we did not know whether the human fetal liver was an appropriate source for EPO-mRNA. Further, immunoprecipitation of EPO using antibodies believed to be EPO specific failed because of the rarity of EPO mRNA and because the antibodies proved to be non-EPO specific. These were among the difficulties or obstacles which caused us eventually to abandon our project. I also know that we were not the only ones to encounter such obstacles in cloning the EPO gene. On several occasions, I had discussions with researchers at Biogen who were also attempting to clone the EPO gene, and they indicated that they had encountered similar problems and obstacles as we had and were equally unsuccessful.

12. From my knowledge of Dr. Lin's success in cloning the EPO gene and as disclosed in the patent EP 148 605, I understand that Dr. Lin used mixtures of fully degenerate probes to screen a human genomic library instead of a cDNA library. To my recollection, this was the first successful use of this mixed probe approach in a genomic library. If I had been asked to evaluate this approach in 1983, I would have considered it not likely to succeed because of the high degree of degeneracy of the limited amino acid sequence available for EPO and the inherent difficulties in probing a genomic library, e.g.,

introns, background, etc. For these reasons, I considered then and still do today Dr. Lin's work to be quite an outstanding achievement.

13. I also would like to respond to some of the specific arguments of Dr. Sytkowski concerning our work related to EPO. First, we abandoned our efforts to clone the EPO gene because we had reached obstacles which we could not overcome. If we had thought that we could have done it, we would have continued our efforts despite rumors that other groups were ahead of us. My nature is to pursue a project to its conclusion rather than wait and let someone else do the work for me as Dr. Sytkowski suggests. Moreover, we had received a grant from the NIH to conduct these studies, and it would have been unfair to the NIH (and the American taxpayer) to simply "wait until one of the companies got it" as Dr. Sytkowski recalls. This was an important project for us and we gave it our best effort.

14. As for Dr. Sytkowski's arguments concerning Dr. Goldwasser and the availability of EPO derived from urinary sources, I need not comment on the apparent friction between Dr. Sytkowski and Dr. Goldwasser, but suffice it to say that Dr. Goldwasser had published his purification scheme in the 1977 Miyake et al. publication (J. Biol. Chem. 252, 5558-64) and as part of our grant study, Dr. Sytkowski had arranged to collect the urine of chronic anemia patients in the Boston area. As recorded in the Project Site Visit Report, p. 6, Dr. Sytkowski had "already collected one million units of erythropoietin for the purification studies and apparently [has] a well organized program to collect large amounts of human urine which would be required for the purification studies." I certainly do not blame Dr. Goldwasser for our failures. Cloning the EPO gene was just a difficult and frustrating problem. Since EPO is such a rare protein, one of the problems was having a sufficient supply of EPO available. A scientist would always like as much material as possible to use in various experiments, but in this case, availability of EPO was not the only problem and even if we had had more EPO, there was no certainty that we would have been successful.

15. Finally, I do not understand Dr. Sytkowski's argument that simply because the NIH study group deemed it reasonable to fund our EPO project that that decision somehow affects the validity of the patent rights on Dr. Lin's invention. While I am not well versed in the legal requirements to obtain a patent, as a research scientist, I would hope that patent rights to an important invention are not preempted before the research is even conducted because of a decision by the NIH that the prospects of success are reasonable enough to fund the project. Also, I know that many factors are considered by the NIH in approving grant proposals that are unrelated to the merits of the particular proposal. For example, with respect to the grant discussed herein relating to EPO, this was a "bundled" proposal that was part of a much larger overall project under the direction of Dr. Nathan. My own project consisted of two parts relating to alpha globin and EPO as explained above. Our group had demonstrated proficiency in the past and had received various grants and awards as a result of that demonstrated record of success. For example, I received the NIH Young Investigator Award for the years 1976-79 and the NIH Research Career Development Award for the years 1979-84. I believe the confidence of the NIH study group in our grant proposal was based more on our past successes rather than strictly on the prospect of success for the EPO project.

16. I would further add that once the EPO gene was cloned and the sequence made available, it was straightforward for someone to clone and express the gene. In our own research, we did just that. Beginning in about the Summer of 1985, we commenced a project to (1) clone the human EPO gene from a genomic library, (2) clone an EPO cDNA, and (3) express the cDNA in insect cells in order to study the N-linked oligosaccharide on recombinant EPO. This work was published in Wojchowski et al, Biochim. Biophys. Acta, 910, (1987), pp. 224-32, (attached as Exhibit D hereto), and Dr. Sytkowski and I were co-authors on this paper. The genomic cloning of the EPO gene and its characterization was performed by Don Wojchowski, who was a post-doctoral Fellow working in Dr. Sytkowski's laboratory, using information from the EPO gene sequence published by Jacobs et al,

Nature, 313 (1985), pp. 806-810. As described in the Wojchowski article, we were successful in cloning the EPO gene from a human genomic library similar to the Lawn library using an oligonucleotide probe corresponding to the first 30 nucleotides of protein coding exon IV from the Jacobs sequence. This sequence is identical to the sequence of exon IV in Table VI of EP 148 605. The genomic cloning was completed and the gene characterized within a few months. Next, we generated EPO mRNA through the transient expression of the human genomic clone in COS cells. We then cloned EPO cDNA as described in the Wojchowski article and expressed recombinant EPO in COS cells and later in insect cells. The cloning of EPO cDNA and expression in COS cells was completed within the first part of 1986, and timewise, I would comment that this project was one of several we were working on at the time, and we did not pursue it at a furious pace. Thus, with the EPO gene sequence available, it was straightforward and only required a few months to clone the gene and to express the recombinant protein.

Executed this 3rd day of January 1997 at Boston, Massachusetts.

Stuart H. Orkin

Stuart H. Orkin

CURRICULUM VITAE

Name: Stuart H. Orkin, M.D.

Home Address: 16 Winnetaska Rd., Waban, MA 02168

Date of Birth: April 23, 1946

Place of Birth: New York, N.Y.

Education:

1967 BS	Massachusetts Institute of Technology, Cambridge, MA.
1972 MD	Harvard Medical School, Boston, MA.

Postdoctoral Training:

Internship and Residency:

1972-73	Intern in Pediatrics, Children's Hospital Medical Center, Boston, MA
1975-76	Resident in Pediatrics, Children's Hospital

Clinical Fellowships:

1972-73	Clinical fellow in Pediatrics, Harvard Medical School
1975-76	Clinical fellow in Pediatrics, Harvard Medical School
1976-78	Fellow in Medicine (Hematology and Oncology), Children's Hospital Medical Center and the Dana Farber Cancer Institute, Boston, MA

Research Fellowships:

1973-75	Research Associate, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, NIH, Bethesda, MD
1976-78	Research Fellow in Pediatrics, Harvard Medical School

Licensure and Certification:

1978	Massachusetts License
1978	American Board of Pediatrics

Academic Appointments:

1978-81	Assistant Professor of Pediatrics, Harvard Medical School
1981-87	Associate Professor of Pediatrics, Harvard Medical School
1986-	Investigator, Howard Hughes Medical Institute
1987-	Leland Fikes Professor of Pediatric Medicine, Harvard Medical School

Hospital Appointment:

1978-	Associate in Medicine (Hematology and Oncology), Children's Hospital Medical Center
1978-	Associate Physician, Dana Farber Cancer Institute

Awards and Honors:

1976-78	Basil O'Connor Starter Research Award of the National Foundation-March of Dimes
1976-79	NIH Young Investigator Award
1979-84	NIH Research Career Development Award (NHLBI)
1984	AFCR Clinical Research Award
1986-	Investigator, Howard Hughes Medical Institute
1986	Dameshek Award, American Society of Hematology
1987	Chairman, Gordon Conference on Molecular Genetics
1988	Mead Johnson Award, American Academy of Pediatrics
1988	Ben Abelson Visiting Professor, Department of Pediatrics, Washington University
1988	Karl Meyer Lectureship, University of California Medical Center
1988	Harvey Lecture
1989-90	Councillor, American Society of Hematology
1989	President, American Society of Clinical Investigation
1991-	Robert and Courtney Steel Visiting Professorship in Pediatrics at Memorial Sloan Kettering Cancer Center
1992-	Member, National Academy of Sciences
1992	Member, Institute of Medicine
1992-	The Stratton Lecture, 24th Congress of the International Society of Haematology
	Member, American Academy of Arts and Sciences

Major Committee Assignments:

National and Regional

1992	Member, External Advisory Review Committee on Gene Therapy, Department of Internal Medicine, The University of Michigan Medical Center
1992-	Awards Committee, E. Mead Johnson Award for Research in Pediatrics
1993	Scientific Advisor, Lindsley F. Kimball Research Institute of the New York Blood Center

1993 External Advisory Committee for Research, The University of Texas, MD Anderson Cancer Center
1993 External Advisory Committee, Indiana University School of Medicine, Herman B. Wells Center for Pediatric Research

Hospital
1990- The Research Faculty Council
1992- Awards and Review Committee

Professional Societies:

1979- American Society of Hematology
1980- Society for Pediatric Research
1982- American Society for Clinical Investigation
1983- American Society of Human Genetics
1989- Association of American Physicians

Editorial Boards:

1983-1986 Blood
1983-1986 American Journal of Human Genetics
1987-1989 Genomics
1990-1991 New England Journal of Medicine
1992- Annual Review of Genetics

Major Research Interests:

1. Molecular genetics and biology of human disease
2. Thalassemia syndromes and hemoglobin synthesis
3. Prenatal diagnosis of genetic disease
4. Molecular biology of coagulation
5. Molecular genetics and biochemistry of the phagocytic cell
6. Gene transfer and expression in hematopoietic cells

Teaching Experience:

1979- Lectures in hematology section of the Harvard Medical School pathophysiology course
1979- Attending physician, Division of Hematology Oncology, Children's Hospital and Sidney Farber Cancer Institute
1980- Attending physician, Department of Medicine, Children's Hospital Medical Center
1979- Lecturer in Harvard Medical Postgraduate Internal Medicine and Pediatric Courses

Administrative Roles:

1985-	Member, MD-PhD Selection Committee (Harvard Medical)
1987-1988	Member, National Research Council Committee on Mapping and Sequencing the Human Genome
1987-1991	Mammalian Genetics Study Section, NIH

Principal Clinical and Hospital Service Responsibilities:

1978-	Attending physician, Division of Hematology Oncology, Children's Hospital and Sidney Farber Cancer Institute
1980-	Attending physician, Department of Medicine, Children's Hospital

Publications

A. Original Reports

1. Dowben, R.M., Orkin, S.H.: Extrinsic cotton effects in dye-bovine plasma albumin adducts. Proc. Natl. Acad. Sci. USA 58: 2051-2054, 1967.

2. Orkin, S.H., Littlefield, J.W.: Nitrosoguanidine mutagenesis in synchronized hamster cells. Exp. Cell Res. 66: 69-74, 1971.

3. Orkin, S.H., Littlefield, J.W.: Mutagenesis to aminopterin resistance in cultured hamster cells. Exp. Cell Res. 69: 174-180, 1971.

4. Orkin, S.H., Buchanan, P.D., Yount, W.J., Reisner, H., Littlefield, J. W.: Lambda-chain production in human lymphoblast-mouse fibroblast hybrids. Proc. Natl. Acad. Sci. USA 70: 2401-2405, 1973.

5. Orkin, S.H., Harosi, F.I., Leder, P.: Differentiation of erythroleukemic cells and their somatic hybrids. Proc. Natl. Acad. Sci. USA 72: 98-102, 1975.

6. Leder, A., Orkin, S.H., Leder, P.: Differentiation of erythroleukemic cells in the presence of inhibitors of DNA synthesis. Science 90: 893-894, 1975.

7. Orkin, S.H., Swan, D., Leder, P.: Differential expression of alpha- and beta-globin genes during differentiation of cultured erythroleukemic cells. J. Biol. Chem. 250: 8753-8760, 1975.

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11. Orkin, S.H.: Selective restriction endonuclease cleavage of human globin genes. J. Biol. Chem. 253: 12-15, 1978.

12. Orkin, S.H., Alter, B.P., Altay, C., Mahoney, M.J., Lazarus, H., Hobbins, J.C., Nathan, D.G.: Application of endonuclease mapping to the analysis and prenatal diagnosis of thalassemias caused by globin gene-deletion. N. Engl. J. Med. 299: 166-172, 1978.

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20. Michelson, A., Orkin, S.H.: The 3'-untranslated regions of the duplicated human alpha-globin genes are unexpectedly divergent. Cell 22: 371-377, 1980.

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deoxynucleotidyl transferase: implications for the cloning of cDNA. J. Biol. Chem. 257: 14773-14782, 1982.

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of the nucleotide which is substituted in sickle cell anemia. Amer. J. Human Genetics, 35: 1028-1033, 1983.

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With the assistance of Dr. Timothy Springer in the Department of Pathology, Harvard Medical School, we have performed several trial fusions of line P3X with both mouse splenic lymphocytes and rat splenic lymphocytes. Briefly, the spleens are harvested aseptically and disaggregated to provide a suspension of lymphocytes. $1x10^8$ lymphocytes are mixed with $1-3x10^7$ log phase myeloma cells and are fused in the presence of polyethylene glycol, PEG according to a modification of the method of Milstein. Suspensions of fused cells are plated into selective medium (HAT) in microtiter wells and incubated at 37°C. In 2-4 weeks colonies of hybrid cells are visible. In our hands between 100-300 colonies are obtained from a typical fusion. Thus we have demonstrated our capacity to both cultivate the appropriate myeloma cells and to fuse them successfully with mouse and rat lymphoid cells resulting in hybrid cell colonies.

Utilizing our own modifications of standard published methods we have developed enzyme-linked immunosorbent assays directed against soluble protein antigens. Protein antigens are adsorbed to polyvinylchloride microtiter dishes. The solid phase protein is then incubated in the presence of serial dilutions of known antisera. In establishing this system we first immunized rabbits with mouse adult hemoglobin as antigen to permit us to assess the sensitivity of this model system. After incubation with diluted antiserum, the wells are washed and horseradish peroxidase conjugated goat antirabbit antiserum (1:1000 dilution) is added and incubation continued for an additional 2 hours. Bound antibody is detected by the addition of substrate (orthophenyldiamine) and hydrogen peroxide. In those wells containing bound antibody, a visible color appears in 5-30 minutes. This ELISA assay is readily applicable to supernatant medium from clones of hybridoma cells as well as the serial dilutions of rabbit antiserum which were employed in setting up the method. Of course, appropriate species-specific second antibody is required.

In order to establish a specific assay for anti-erythropoietin antibodies our initial intent was to bind purified erythropoietin antigen to polyvinylchloride wells as discussed above and employ either an enzyme-linked immunosorbent assay or a radioimmunosorbent assay to detect bound antibodies. However, to date we have been unable to obtain purified erythropoietin from the NHLBI committee responsible for its distribution. Since homogeneous erythropoietin is not yet available to us for this purpose we developed an immunoprecipitation assay as follows. Known concentrations of partially purified human erythropoietin are incubated in the presence of serial dilutions of rabbit anti-erythropoietin antiserum. After a suitable time the antigen-antibody complexes are precipitated with goat anti-rabbit antiserum. The supernatant medium from this immunoprecipitation is depleted of erythropoietin in proportion to the amount of anti-erythropoietin antibody present in the original incubation. This supernatant is then subjected to erythropoietin bioassay by our highly sensitive cryopreserved rabbit bone marrow technique. Although laborious and time-consuming we have shown this technique to be quite sensitive and highly specific for antibodies directed against biologically active erythropoietin. We believe that this method, which is presented in outline form here, can be directly transferred to our screening of hybridoma supernatants for either rat or mouse IgG directed against human urinary erythropoietin. Obviously this immunoprecipitation method would be simpler if ^{125}I-erythropoietin were available. However since the purified starting material was not made available to us we must at present rely upon our immunoprecipitation bioassay for screening these hybridoma clones.

3. Goals for the 02 Year

Since we now have on hand all the techniques necessary to pursue our primary objective - the preparation of monoclonal antibodies to erythropoietin - the 02 year will see an all-out effort directed toward this end. We have already begun to immunize both rats and mice with partially purified erythropoietin derived in our laboratory. We are utilizing several immunization schedules and will soon be in the position to screen these animals for the presence of anti-erythropoietin antibodies in their serum utilizing the above described technique. Simultaneously we are continuing our efforts to

purify the hormone so that we may prepare our own homogeneous antigen for use in a simpler ELISA assay. As soon as we are successful in identifying immunized animals with anti-erythropoietin titers, we will proceed with fusion and hybridoma generation as described above. While this process is ongoing we propose to explore the biochemical methods necessary to develop immunoaffinity columns directed against erythropoietin. Employing heterologous anti-erythropoietin antiserum made available to us as well as antiserum which we will generate in rabbits we will purify the IgG fraction and covalently link it to a solid support, viz., Sepharose beads. We shall then explore methods of application of erythropoietin preparations to such antibody columns permitting the total adsorption of the hormone by the antibodies and, thereafter, devise methods of elution of the antibody-bound erythropoietin so as to achieve maximal yield and simultaneously preserve biological activity. We believe that it is necessary to explore these methods well in advance of the availability of monoclonal antibodies to erythropoietin. When these monoclonal antibodies are on hand we will be ready to move rapidly toward an affinity purification of the hormone.

III. Project III: Molecular Aspects of Globin Gene Switching and Cloning of Erythropoietin Sequences

Project leader: Stuart H. Orkin, MD

A. Publications During the 01 Year

1. Orkin SH, Goff SC: The duplicated human α-globin genes: Their relative expression as measured by RNA analysis. Cell 24:345-351, 1981
2. Michelson AM, Orkin SH. J Biol Chem (submitted, 1982)

B. Personnel

1. Additions: David Ginsburg, part-time research associate
   Ann Durso, part-time secretary
2. Deletions: Edward Prochownik, MD, research associate

C. Changes in Effort

1. Until our monospecific antibodies to erythropoietin (to be prepared by A. Sytkowski) are available for cloning studies, we will concentrate on further development of cDNA cloning and expression of the cloned molecules in E. coli. When the antibodies are ready, they can be quickly used for isolation of the appropriate clones.
2. Similarly, until purified erythroid populations are available (from D.G. Nathan's section) we will concentrate on trying to examine ζ and α gene expression in early embryonic material.

D. Progress Report

1. Goals for the 01 Year

a) Study of expression from the α gene complex in early erythroid development.
b) Investigation of possible approaches to cloning of erythropoietin RNA sequences.
c) Establishment of cDNA cloning methodologies and expression of cDNAs in E. coli.

2. Progress in Achieving Goals for the 01 Year

(a) Expression from the α gene complex

Our initial studies (ref. 1) established the techniques for distinguishing the mRNAs transcribed from the duplicate human α-globin genes. These showed that expression of the more 3' gene (α2) into stable mRNA exceeds that of the 5' gene (α1). Our main concern is whether the relative expression of these genes is fixed, especially during early fetal or embryonic life when switching from ζ to α gene expression occurs. So far, we have studied samples as early as only 10 weeks fetal life. We have attempted, so far unsuccessfully for technical reasons, to analyze earlier samples provided

by D. Linch and his colleagues in London. Further analysis of additional samples should permit progress in this area. We have examined one embryonic-like cell, the K562 erythroleukemia line, and found surprisingly a vast predominance of the 21 mRNA. Whether this is a normal pattern during early development awaits further study of early fetal samples.

(b) Approaches to cloning of erythropoietin sequences

We have pursued two approaches to this problem. First, we have asked what the most favorable tissue sample might be. We have investigated hypoxic rat and primate kidney samples as well as human fetal liver. Rat kidneys were prepared by M. Miller at Brookhaven Laboratories and baboon kidney by J. DeSimone at Chicago. In the latter instance, animals were kept under severe chronic hypoxemic stress to augment erythropoietin production greatly. Radioimmunoassay of tissue and serum samples by J. Garcia at Lawrence Laboratories in Berkeley, CA, showed that our baboon serum and tissue specimens are $>10^3$ times basal erythropoietin levels. Rat kidney and human fetal liver are somewhat increased but not to the same extent. Therefore, we believe that the hypoxemic baboon tissue that we have constitutes a greatly enriched source of erythropoietin mRNA in all likelihood. Second, we have prepared undegraded mRNA from the baboon tissue using a guanidine HCl extraction and translated it in vitro in the rabbit reticulocyte lysate system. Translation products were immunoprecipitated with heterospecific anti-Epo antibody (100 U/ml) provided to us by J. Garcia. Unfortunately, numerous kidney proteins were precipitated at various concentrations of antibody and after various preadsorptions. Therefore, the lack of specificity of this antibody preparation precluded identification of translated erythropoietin mRNA. In addition, samples of various translation mixtures containing putative Epo-mRNA were subjected to radioimmunoassay by J. Garcia. No Epo above background was detected. However, the sensitivity of the assays was insufficient to assure detection of the levels of protein likely to be synthesized in vitro.

(c) Cloning of cDNAs and their expression in E. coli

Cloning of erythropoietin mRNA sequences requires efficient methods of cDNA cloning. During the 01 year we have adapted previous methods of cDNA cloning. Using conventionally prepared cDNA, attention to complete copying into a second strand with DNA polymerase (Klenow fragment), and modified terminal transferase tailing of cDNA, we now generate about 10^5 clones/ug of mRNA. This is sufficient to generate complete libraries of any tissue mRNA population. We have constructed initial liver libraries from human adult and fetal samples in the plasmid pKT218 and are investigating expression of specific cDNAs in E. coli.

IV. Project IV: The Development of Red Cell Membrane Proteins

Project leader: Samuel E. Lux, MD

A. Publications During the 01 Year

1. Spiegel JE, Beardsley DS, Lux SE: Identification of a protein in nonerythroid human cells which cross-reacts with erythrocyte protein 4.1. Fed Proc 41:657, 1982 (abstr)
2. Becker PS, Spiegel JE, Lux SE: A new procedure for high-yield purification of protein 4.1 from human red cell membranes. Fed Proc 41:657, 1982 (abstr)

B. Personnel

1. Additions: Judith Spiegel, research fellow
   Ann Durso, secretary (25%)
2. Deletions: Clinton Joiner, MD, research associate

C. Changes in Effort: None

D. Progress Report

1. Goals for the 01 Year

Our work in the 01 year was focused on the development of specific antibodies to red cell membrane proteins, particularly membrane skeletal proteins, and the application

Natha David G. 5 HL 27375-02

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ordinary CFU-E will then be exposed to increasing concentrations of erythropoietin and BPA to determine whether the low output of HbF in the colonies to which they give rise represents the result of their own immutable gene program and is not a function of the environment in which the cultures are actually grown.

(c) Effects of BPA:

The effects of burst promoting activity on fetal hemoglobin synthesis described above must be pursued because this experiment is the only one, which has to our knowledge, evoked a change in fetal hemoglobin synthesis without loss of hemoglobin production in culture. It is imperative to determine whether this finding has therapeutic significance. Can lymphokines induce fetal hemoglobin synthesis? To approach this, we will repeat the rhesus findings in Cebus monkeys or marmosets now available to us in the School of Public Health at Harvard University. If erythroid progenitors of these much smaller simians also demonstrate the capacity to produce fetal hemoglobin in culture and demonstrate as well an increment in fetal hemoglobin when burst promoting activity is added to culture, we will then begin an attempt to treat such monkeys with burst promoting activity following sufficient bleeding to induce BFU-E terminal differentiation. This will permit us an opportunity to approach the problem in vivo, and finally determine whether lymphokines can in fact modulate γ chain production in a potentially therapeutic manner.

II. Project II: Erythropoietin and Anti-Erythropoietin Antibodies

Project leader: Arthur J. Sytkowski, MD

A. Publications During the 01 Year

None

B. Personnel

1. Additions: Karen L. Bicknell, research technician
Rena Bacon, research associate scheduled to start January, 1985.
Ann Durro, part-time secretary
2. Deletions: John N. D'Albis, research technician

C. Changes in Effort: Arthur J. Sytkowski reduced to 35%.

D. Progress Report

1. Goals for the 01 Year

Our work in the 01 year has emphasized the development of monoclonal antibody technology. This effort is directed toward the ultimate preparation of monoclonal antibodies to human urinary erythropoietin. Specific scientific objectives included: a) the acquisition of mouse myeloma cell lines and the cultivation of these cells in vitro; b) the derivation of techniques to permit fusion of these myeloma lines with immune lymphoid cells from the spleens of mice (allogeneic hybridization) and rats (xenogeneic hybridization); c) the development of a solid phase enzyme linked immunosorbent assay (ELISA) for soluble protein antigens; d) development of a specific assay for anti-erythropoietin antibodies.

2. Progress in Achieving Goals for the 01 Year

We have obtained two mouse myeloma lines deficient in hypoxanthine-guanine-phosphoribyltransferase (HGPRT). Both are nonimmunoglobulin producing. They are line SP2/0 and line P3X63Ag3653 which were obtained from the Human Genetic Mutant Cell Repository at the Institute for Medical Research in Camden, NJ. These lines have been demonstrated to exhibit a high fusing capacity in other laboratories. The cells are grown in 90% Dulbecco's Modified Eagle medium enriched with glucose, pyruvic acid, oxalacetic acid, inculin, and 10% fetal calf serum in a humidified atmosphere of 90% air/10% CO_2. Both lines demonstrate greater than 85% viability in our laboratory and both have been cryopreserved.

Name of PI/PD/Program Coordinator or Co-Associate (Last, first, middle)
Nathan, David G.

Social Security Number
022-22-5319

(b) The Simian System

During the 02 year we completed studies of the fetal hemoglobin program in simian progenitors and showed clearly that the F-program is a function of progenitor maturity. In addition we found that in the most mature progenitors, termed a HERCs, the F-program is completely extinguished. Comparison studies of human erythropoiesis were also completed during the 02 year and showed substantially different results. In humans the F-program is constant through the CFU-E and hemoglobin F synthesis is not extinguished until the level of the proerythroblast. In other studies we found that there was no apparent influence of azacytidine on this program in vitro.

Studies of erythropoiesis in the simian fetus were continued during the 02 year. The evidence to date suggests that two progenitors are present - one fetal and one adult.

3. Goals for the 03 Year

(a) We plan to develop a simian model that will allow us to investigate the effects of chemotherapeutic agents on hemoglobin F synthesis.

(b) We also plan to investigate the F-program in thalassemia and sickle cell disease using patients with a high level of hemoglobin F (thal intermedia or SS with high F) to determine whether the high concentration of hemoglobin F is due to selection or to an increase in the F-program.

II. Erythropoietin and Anti-Erythropoietin Antibodies

Project Leader: Arthur J. Sytkowski, M.D.

A. Publications during the 02 Year

Sytkowski AJ, Richie JP, Bicknell KA. A new human renal carcinoma cell line established from a patient with erythrocytosis. Cancer Research 1983; 43:1415-1419.

Sytkowski AJ, Perrine SP, Bicknell KA, Kessler CJ. Erythropoietin-induced differentiation of rauscher erythroleukemia cells. Proceedings of the Third Conference on Hemoglobin Switching (In Press).

Sue JM and Sytkowski AJ. Site-specific antibodies to human erythropoietin directed toward the NH_2-terminal region. Proc. Natl. Acad. Sci. USA (In Press).

B. Personnel

1. Additions: None
2. Deletions: None

C. Changes in Effort: Dr. Rena Bacon, Research Associate, increase to 100% effort.

Name (If PD/Program Coordinator or Candidate (Last, first, middle)
Nathan, David G.

Social Security Number
022-22-5319

D. Progress Report

1. Goals for the 02 Year During the 02 year we planned to:

(a) prepare monoclonal and polyclonal antibodies to erythropoietin,

(b) continue our efforts to purify erythropoietin, focusing particularly on immunoaffinity, chromatography,

(c) develop an immunoassay for the hormone.

2. Progress in Achieving Goals for the 02 Year

(a) Preparation of antibodies to erythropoietin

Considerable success was achieved on this aspect of the project during the 02 year. This was accomplished using a new approach to the preparation of erythropoietin antibodies. A synthetic peptide was prepared containing the first 26 amino acids of the N-terminal sequence of human erythropoietin recently reported by Goldwasser. This peptide was attached to albumin and the complex was used to immunize rabbits. The resulting polyclonal antibodies were purified on an albumin-affinity column. This antibody precipitated over 90% of biologically active erythropoietin (assessed using a rabbit bone marrow assay). In addition, the antibody precipitated purified ^{125}I-erythropoietin (obtained from Garcia and Clemons). SDS gels of the immunoprecipitate disclosed a 39,000 dalton radioactive band, which corresponds to the published molecular weight of erythropoietin. In other studies we show that either unlabeled synthetic peptide or unlabeled, biologically active crude erythropoietin could compete with purified ^{125}I-erythropoietin for the antibody. The K_i's for both the peptide and biologically active erythropoietin were 3×10^{-8} M.

(b) Purification of Erythropoietin

No progress was made on this goal during the 02 year; however, with the availability of the antibody to the synthetic erythropoietin peptide we hope to make substantial progress toward this objective during the 03 year.

(c) Immunoassay for Erythropoietin

The antibody to the synthetic erythropoietin peptide was used to established the radioimmunoassay. One of the greatest advantages of this assay is that ^{125}I-synthetic erythropoietin peptide (large amounts available) can be used in place of purified ^{125}I-erythropoietin(rare and precious). The assay was standardized against biologically active erythropoietin and against the International Reference Standard.

3. Goals for the 03 Year

(a) To adapt the erythropoietin RIA for clinical use.

(b) To make monoclonal antibodies, especially high-affinity monoclonals to the synthetic erythropoietin peptide.

Name of PI/PD/Program Coordinator or Candidate (Last, First, Middle): Nathan, David G.

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(c) To use the polyclonal and monoclonal erythropoietin antibodies to purify human urinary erythropoietin.

(d) To begin studies, using these antibodies, to isolate the erythropoietin gene (in collaboration with Dr. Stuart Orkin).

III. Project III: Molecular Aspects of Globin Gene Switching and Cloning of Erythropoietin Sequences

Project Leader: Stuart H. Orkin, M.D.

A. Publications During the 02 Year

Michelson AM, Orkin SH: Characterization of the homopolymer tailing reaction catalyzed by terminal deoxynucleotidyl transferase: implications for the cloning of cDNA. J Biol Chem 1982; 257: 14773-14782.

B. Personnel

Additions: None
Deletions: David Ginsburg

C. Changes in Effort: Sabra Goff, Research Associate, effort decreased from 60% to 20%

D. Progress Report

1. Goals for the 02 Year

To continue efforts to clone erythropoietin gene sequences.

2. Progress in achieving goals for the 02 Year

During the 02 year we continued our efforts to clone erythropoietin gene sequences. Attention was directed to two tissue sources for cloning: human fetal liver and hypoxic baboon kidney RNA. cDNA libraries were constructed in a pBR322 derivative and were screened with oligonucleotide probe mixtures directed to the N-terminal peptide sequence of erythropoietin reported by Goldwasser. We found fetal liver clones that hybridized to these oligonucleotides but DNA sequencing showed that these were not erythropoietin. Studies of cDNA colonies isolated from baboon kidney gave similar results. The most likely explanation is that the portion of the amino acid sequence used to construct the oligonucleotide probes (the C-terminal end of the 28 residue sequence) is incorrect.

3. Goals for the 03 Year

Work during the 03 year will focus on the use of additional oligonucleotide probe mixtures prepared to the N-terminal end of the Goldwasser sequence and on expression of cloned sequences in E. coli.