Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed 56-character length restrictions, including spaces.

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT
Mechanism of Square Cell Disease in the Kidney

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
(If "Yes," state number and title)
Number: K08 Title: "PA-00-003 MENTORED CLINICAL SCIENTIST DEVELOPMENT AWARD."

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

New Investigator ☐ **No** ☒ **Yes**

3a. NAME *(Last, first, middle)*
LAWRENCE, Wayne S.

3b. DEGREE(S)
B.S. M.D.

3c. POSITION TITLE
Sr. Fellow/Acting Instructor

3d. MAILING ADDRESS *(Street, city, state, zip code)*
University of Wenatchee
Div. of Renal Medicine
800 Entiat River Road
Wenatchee
WA 99201

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Medicine

3f. MAJOR SUBDIVISION
School of Medicine

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 509-884-7173 FAX: 509-884-7172

E-MAIL ADDRESS: LawrenceW@UWEntiat.edu

4. HUMAN SUBJECTS RESEARCH
☐ No
☒ Yes

4a. Research Exempt ☒ No ☐ Yes
If "Yes," Exemption No.

4b. Human Subjects Assurance No.
M1209

4c. NIH-defined Phase III Clinical Trial
☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval Date
12-01-2001

5b. Animal welfare assurance no
A4010-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
12-01-02	11-30-07	$118,970	$128,488	$594,850	$642,438

9. APPLICANT ORGANIZATION
Name University of Wenatchee
Address 800 Entiat River Road
Wenatchee
WA 99201

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
55-0410
DUNS NO. *(if available)*

Congressional District 11th

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Priscilla Levenworth
Title Director, Grant and Contract Services
Address 800 Entiat River Road
Wenatchee
WA 99201

Tel 509-884-7173 FAX 509-884-7172
E-Mail levenwp@uwentiat.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Priscilla Levenworth
Title Director, Grant and Contract Services
Address 800 Entiat River Road
Wenatchee
WA 99201

Tel 509-884-7173 FAX 509-884-7172
E-Mail levenwp@uwentiat.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. 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.

SIGNATURE OF PI/PD NAMED IN 3a.
(In ink. "Per" signature not acceptable.)

DATE
1/10/02

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13.
(In ink. "Per" signature not acceptable.)

DATE
1/19/02

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Although renal complications are the leading cause of death in square cell disease, the kidney has rarely been the focus of basic research in this disorder. Current understanding of square cell pathophysiology derives from studies performed in vitro or in other organs. Because mechanisms of vaso-occlusion and inflammation in the kidney are likely to be different from those of other organs, there is a critical need for basic square cell research that focuses on the kidney. The proposed research will investigate mechanisms of renal vaso-occlusion and kidney inflammation in animal models of square cell disease. In specific aim 1, I will use isolated rat kidneys perfused with erythrocytes from patients with square cell disease to identify determinants of renal vaso-occlusion. Renal hemodynamics and kidney sequestration of radiolabeled erythrocytes will be used as surrogate markers of vaso-occlusion. This model will be used to evaluate the importance of tubular hypertonia, mixed arterialhypertonia, inflammation, and erythrocyte-endothelial adhesion to vaso-occlusion in the kidney. In specific aims 2 and 3, I will use a transgenic-knockout mouse model of square cell disease ("square cell mice") to investigate mechanisms of kidney inflammation in square cell disease. In specific aim 2, square cell and control mice will receive intravenous infusion of hypertonic saline and the leukocyte, cytokine and chemokine responses will be measured in blood. kidney. and tubular fluid. Activation of renal rnicrovascular endothelium in these mice will be assessed with immunohistochemistry. Blocking antibodies to c5b1 will be used to determine if cellular adhesion contributes to kidney inflammation in square cell mice after hypertonia-induced vaso-occlusion. In specific aim 3, I will test the hypothesis that square cell mice exhibit an enhanced inflammatory response to lipopolysaccharide, suggesting increased susceptibility to acute kidney injury. Mice will receive intravenous infusion of emulsified lipopolysaccharide and leukocvte, cytokine, and chemokine responses will be measured in blood, kidney, and tubular fluid. Blocking antibodies to CD99 will be used to determine if an anti-neutrophil strategy attenuates kidney inflammation to a greater extent in square cell than control mice. These experiments will improve understanding of vaso-occlusion and inflammation in the kidney, and ultimately lead to new treatments for patients with renal complication.

PERFORMANCE SITE(S) *(organization, city, state)*

University of Wenatchee
Department of Medicine
Laboratory of Renal Medicine
800 Entiat River Road
Wenatchee
WA 99201

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Lawrence, Wayne, M.D.	U of Wenatchee	PI
Lou, McKinzie, M.D.	U of Wenatchee	Sponsor
Gertrude, Alice, M.D.	Sequim Medical Resh. Center	Co-Sponsor
Dale, Elmond, M.D.	U of Wenatchee	Collaborator
Terry, Annabel, M.D.	Queets Resh. Center.	Collaborator

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ **Yes** ☐ **No**

RCA TOC Substitute Page Candidate *(Last, first, middle):* Lawrence, Wayne S.

Use this substitute page for the Table of Contents of Research Career Awards. The name of the candidate must be provided at the top of each printed page and each continuation page.

RESEARCH CAREER AWARD
TABLE OF CONTENTS
(Substitute Page)

8. Appendix *(Five collated sets. No page numbering necessary)* ☐ Check if Appendix is included

Number of publications and manuscripts accepted or submitted for publication *(not to exceed 6)* 0

List of Key Items:

renal
kidney
square cell disease
inflammation

Note: Type density and size must conform to limits provided in the Specific Instructions.

*Include these items only when applicable.

CITIZENSHIIP

☒ U.S. citizen or noncitizen national ☐ Permanent resident of U.S. (If a permanent resident of the U.S., a notarized statement must be provided by the time of award.

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD *(from Form Page 4)*	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*	99,692	99,692	99,692	99,692	99,692
CONSULTANT COSTS					
EQUIPMENT	761				
SUPPLIES	11,419	12,179	12,179	12,179	12,179
TRAVEL	1,999	1,999	1,999	1,999	1,999
PATIENT CARE COSTS — INPATIENT					
PATIENT CARE COSTS — OUTPATIENT					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES	5,099	5,099	5,099	5,099	5,099
SUBTOTAL DIRECT COSTS	118,970	118,970	118,970	118,970	118,970
CONSORTIUM/ CONTRACTUAL COSTS — DIRECT					
CONSORTIUM/ CONTRACTUAL COSTS — F&A					
TOTAL DIRECT COSTS	118,970	118,970	118,970	118,970	118,970

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face Page)* **$** 594,850

SBIR/STTR Only Fee Requested					

SBIR/STTR Only: Total Fee Requested for Entire Proposed Project Period **$**

(Add Total Fee amount to "Total direct costs for entire proposed project period" above and Total F&A/indirect costs from Checklist Form Page, and enter these as "Costs Requested for Proposed Period of Support on Face Page, Item 8b.)

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel: Dr. Lawrence will commit 80% effort to the proposed project, receiving $75,000 per year plus benefits at the rate of 25.3%. Peter Tsai, a research technician I will devote 15% of his time to the project, assisting with preparation of solutions and assisting with animal experiments.

Consultant Costs: None.

Supplies: Costs of antibodies and ELISA kits represent the bulk of these costs. Routine laboratory supplies (reagents, tubing, syringes, pipettes, etc.) make up the remainder of supply costs.

Travel: Funds are requested for two national meetings because the interdisciplinary nature of the research suggests results should be presented to the renal research community and the square cell disease community. In addition, exposure to scientists / idea in both renal and square cell fields is ideal for Dr. Lawrence's development as an expert in square cell - associated renal disease.

Equipment: Funds are requested for a pH meter to be used in isolated kidney experiments.

Other Expenses: Funds to pay human volunteers for participation in the study and for complete blood counts are included in this category. Costs of rat and wild-type mice are also budgeted for the project period.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
LAWRENCE, Wayne S.	Sr. Fellow/Acting Instructor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Wenatchee Valley College, Wenatchee, WA	BS	1987	Biology
University of Washington, Sch. Med. Seattle	MD	1993	Medicine
Johns Hopkins U. School Medicine, Baltimore	Internship	1993-94	
University of Wenatchee, Wenatchee, WA	Residency	1994-95	
University of Wenatchee, Wenatchee, WA	Chief Resident	1996-97	Renal Medicine
University of Wenatchee, Wenatchee, WA	Sr. Fellow/ Acting Instr.	1997-pres	

RESEARCH EXPERIENCE:

1993-1994 Johns Hopkins U. Sch. Med., Human Physiology Laboratory: Training Effects of Ultradistance Running in Triathletes.
1995 University of Wenatchee; Renal Complications of Square Cell Disease.

HONORS AND AWARDS:

1986 Biology Teaching Award for Teaching Assistants, Wenatchee Valley College
1992 Alpha Omega Alpha, School of Medicine, Univ. Washington, Seattle
1993 Hugo Sorensen Award for Academic Excellence, Univ.Washington, Sch. Med.
2000 Individual NSRA (1 F32 DK10586) Regional Renal Vascular Permeability and Blood Flow in Endotoxemia
2001 Stevens Scholarship, University of Wenatchee, School of Medicine

BOARD CERTIFICATION:

1994 Diplomate of the National Board of Medical Examiners
1996 Diplomate in Internal Medicine, American Board of Internal Medicine
1999 Diplomate in Renal Disease, American Board of Internal Medicine
2000 Diplomate in Critical Care Medicine, American Board of Internal Medicine

CLINICAL / TEACHING ACTIVITIES

1986-1987 Teaching Assistant, Biology 301, Vertebrate Structure and Physiology, with Professor James Henry, Wenatchee Valley College

INVITED LECTURES

High resolution measurments of renal blood flow, NIH Clinical Center, 2000

PROFESSIONAL LICENCES: State of Washington Medical License, (1993-present)

PROFESSIONAL MEMBERSHIPS : American Renal Society, Alpha Omega Alpha

PEER-REVIEWED ORIGINAL RESEARCH:

LAWRENCE, WAYNE S., WA Jones, C Schmeelberg, A. Gertrude, M. Lou. Endotoxemia increases relative perfusion to cortico-medullary kidney regions. Am. J. Applied Physiology, 2002 (in Press)

LAWRENCE, WAYNE S., E. Dale, A. Terry, A. Gertrude, M. Lou. Correlation between diuresis and blood pressure determines filtration/resorbtion heterogeneity in endotoxemia. J. Applied Physiology 89:1339-1351, 2001

LAWRENCE, WAYNE S., W. Erdman, JB Whipple. Effects of prior exercise on renal fluid retention during high intensity exercise in humans. J. Applied Physiology 79:(2) 199-207, 1997

MANUSCRIPTS IN PROGRESS:

LAWRENCE, WAYNE S., M. Lou. Renal protein leak during endotoxemia is heterogeneous but not correlated with regional blood flow.

LAWRENCE, WAYNE S., M. Lou. Sources of Error in measurement of regional renal protein leak.

ABSTRACTS PRESENTED AT NATIONAL MEETINGS

LAWRENCE, WAYNE S., M. Lou. Renal protein leak during endotoxemia in heterogeneous but not correlated with regional blood flow. Am. J. Renal and Critical Care Med. 262(4):A814, 2001

LAWRENCE, WAYNE S., M. Lou. Decreased pressure-filtration matching results from partially marched changes in regional filtration/resorbtion in porcine endotoxemia.

LAWRENCE, WAYNE S., W. Erdman, JB Whipple. Effects of prior exercise on renal fluid exchange kinetics during high intensity exercise in humans. Med. Sci. Sports Exerc. 33(2):S98, 1991

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Lou, McKinzie, M.D.	Associate Professor of Medicine and of Physiology and Biophysics

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Duke University, Durham, NC	B.S.E	1975-1979	Biomedical Engineering
Duke University; Durham, NC	M.A.	1979-1980	Computer Sciences
University of Virginia, Charlottsville, VA	M.D.	1984-1987	Medicine
Duke University; Durham, NC	Residency	1984-1987	Internal Medicine
University of Wenatchee, Wenatchee, WA	Post. Doc	1987-1980	Renal and Critical Care

Comments to share in review discussion:
The mentor, Dr. McKINZIE LOU, has trained several fellows within the Division of Renal and Critical Care Medicine at the University of Wenatchee beginning in 1994. Dr. LOU is a recognized expert in the area of renal blood flow physiology. He is an Associate Professor of Medicine and of Physiology and of Biophysics. He is committed to the training of Dr. LAWRENCE and to the development of this applicant as an independent scientist.

Professional Experience
1984-present: Dr. Lou has followed a traditional training and career path, including a visiting professorship at the Karolinska Institute, and is currently head of renal physiology research training at Univ. Wenatchee.

Publications:
Dr Lou is a very productive researcher, with over 80 refereed publications since 1987, and 40 publications related to these mentored studies over the last 10 years, mostly in the Journal of Applied Physiology, and many dealing with mathematical models of renal perfusion, and most as first or senior author.

Honors , Awards, Service
He has served on many editorial boards and scientific meeting planning committees, has served as an Am Heart Assoc. and NIH grant reviewer, and has won several grants to fund his research over the years.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Gertrude, Alice, MD	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Macalester College, St Paul, MN	BA	1969	Chemistry
U. Pennsylvania, Philadelphia, PA	MD	1973	Medicine
U. Pennsylvania, Philadelphia, PA	Residency	1973-77	Internal Medicine
U. of Wenatchee	Fellowship	1978-80	Renal/Critical Care

Dr. Alice GERTRUDE will provide special training in the area of molecular biology and cytokine and chemokine research.

FACULTY AND HOSPITAL POSITIONS
Dr. Gertrude advanced from 1980 to 1992 from Instructor to Professor of Internal Medicine at U. Wenatchee School of Medicine; becoming director of the Renal Research Training Program in 1993, and in 2000 becoming the Vice Chair, Dept. Medicine, at the Sequim Hospital and Research Center, adjacent to the campus of the U. Wenatchee Medical School.

SPECIAL RESPONSIBILITIES:
Dr. Gertrude has served on many editorial boards and held many Renal Society positions, and has served as an NIH study section reviewer

HONORS AND AWARDS:
Dr. Gertrude was Magna Cum Laude (1969) and Alpha Omega Alpha (1971) and has been awarded academic achievement awards and several NIH grants, with continuous funding since 1985

RELEVANT MAJOR PUBLICATIONS:
Have been 126 since 1980, with 40 relevant to the current application from 1987 to present, mainly in peer-reviewed clinical and immunology journals.

RESOURCES

LABORATORY

Dr. Lawrence will have access to the 843 square foot John Entiat Physiology Laboratory located one floor below his office. The lab is continuously staffed by a veterinarian and two fulltime research assistants funded from other sources. Dr. Lawrence will also use the 225 square foot Integrative Physiology Lab located two floors below his office.

Training in cellular / cytokine physiology and immunohistochemistry will take place in Dr. Gertrude's laboratory. Dr. Gertrude's laboratory consists of 1,600 sq. ft. in five rooms at the Sequim Hospital and Research Center and is staffed by four full-time research technicians, one Ph.D., and one veterinarian in addition to four post-doctoral MDs.

Embedding and sectioning of fixed tissue for histology will be performed in the histology laboratory funded by Dr. Leonard Diener's program project grant. Processing of histological specimens will be performed by Peter Tsai, a research technician employed by the Renal Division and paid by Core 2 of the Program Project Grant.

Mice will be infused with emulsified lipopolysaccharide (LPS) in the vivarium at Seqium Medical Center (the adjacent, University-affiliated County Hospital) using a dedicated programmable microprocessor-controlled syringe belonging to Dr. Alice Gertrude. Mice will be dialyzed against hypertonic saline using an Alzet micro dialysis unit located in the vivarium at the University Of Wenatchee. Dr. Lawrence has full access to these apparati.

CLINICAL

Nurse coordinators from hematology clinics at the University of Wenatchee medical Center, (Doreen Ellis, R.N.) and Sequim Medical Center (Maynard Michalis, R.N.) will assist in identifying patients with square cell disease willing to donate blood. Complete blood counts and differentials on blood from human volunteers will be performed in the clinical hematology laboratory at the University of Wenatchee.

ANIMAL

The animal facilities at the University of Wenatchee are supervised by the Department of Comparative Medicine and are AAALAC –accredited. Facilities and animal husbandry are in compliance with standards outlined in the NIH publication "Guide for the care and Use of Laboratory Animals." Transgenic –knockout square cell mice will be bred in specific-pathogen-free environment in the University Medical Center complex by my collaborator Dr. Elmond Dale.

COMPUTER

Dr. Lawrence has an Apple G4 workstation for his sole use. His computer has high speed internet access and is equipped with software including Statview, Excel, Powerpoint, Word, Kaleidagraph, and Canvas. The entire computer network is backed up daily. A full-time computer programmer funded through the Program Project funded through the Program Project is also available to Dr. Lawrence.

OFFICE

Dr. Lawrence has office space in the Division of Renal and Critical Care Medicine at the U. of Wenatchee Medical Center including personal computer, telephone, voice and e-mail accounts, photocopier, and printer. His office is two floors directly above Dr. Lou's and the Division's administrative offices.

OTHER

Biostatistical consultation is available from Narissa Polestar, Ph.D., Dept. Biostatistics, who is funded through Dr. Leonard Diener's program project grant. General support facilities of the University of Wenatchee Medical Center are available including library, electronic shop, machine shop, medical illustration, audio-visual support, and computer support.

CANDIDATE'S STATEMENT

A. BACKGROUND

My first experience in biomedical research was in the Human Performance Laboratory of John B. Whipple at the University of Washington School of Medicine. We showed that renal fluid exchange was speeded up during high intensity exercise, suggesting that secondary regulators of renal perfusion were operating independently of renal artery pressure in athletes. This research resulted in one publication and one presentation at a national meeting and in my being awarded the Hugo Sorensen Award for Academic Excellence. I have maintained a strong interest in research that has sustained me through my subsequent year of medical training.

Following my four years of residency training in internal medicine and 1.5 years of clinical training in renal/critical care medicine, I have just completed my third full year in the laboratory of Dr. McKinzie Lou. Dr. Lou is currently the mentor for my National Research Service Award, and is primary memtor for my K08 research and training plan. My focus in Dr. Lou's laboratory has been spatial heterogeneity of acute kidney injury. I initially used fluorescent microsphere methods developed by Dr. Lou to study renal perfusion/filtration matching in porcine models of septic shock. This work led to two first author publications. The first showed that endotoxemia increases relative perfusion to cortico-medullary kidney regions. Our analysis suggests that P/F matching is abrogated in toxic shock. The second publication showed an important correlation between diuresis and blood pressure in the determination of filtration/resorbtion and heterogeneity of perfusion in septic shock. These topographical differences suggest there is spatial heterogeneity in the distribution of cellular or molecular mediators of renal perfusion.

Although regional difference in renal perfusion have well-recognized implications for the efficiency of urine formation, little is known about their importance in the development of acute kidney injury. I therefore investigated the relationship between regional differences in renal perfusion and local glomerular capillary barrier function, an indicator of renal injury. Working with Graham Michaels, M.D., Ph.D., Division of Nuclear Medicine (currently University of Ohio), I devel[oped methods to measure regional endothelial leak of protein in the pig kidney, and examined the relationship between regional glomerular leak and local renal blood flow in porcine endotoxemia. This work showed that there are spatially organized regional differences in renal protein leak, but that spatial heterogeneity is not explained by regional differences in renal perfusion. As a consequence of these findings, I now believe that the regional differences in renal protein leak are determined by regional differences in molecular mediators of renal glomerular function. One first author manuscript from this work is nearly complete and another is in progress.

Taken together, my research to date under Dr. Lou's mentorship demonstrates a gradual progression towards independence, and a recognition that cellular/molecular methods must be integrated with standard physiological approaches to determine mechanisms of disease. In addition, my previous research experience demonstrates an ability to work successfully with investigators outside the Renal Division, and a willingness to learn and develop new methodologies.

B. CAREER GOALS AND OBJECTIVES

My career goal is to become an independently funded integrated physiologist with a special expertise in basic mechanisms of square cell disease pathobiology in the kidney. Several factors have led me to redirect my research focus and begin studying square cell kidney disease. First, Square cell kidney disease is an important clinical problem that has attracted few researchers with renal training. Despite its clinical importance, the vast majority of basic square cell disease research has focused on events in the systemic circulation or in vitro models. Thus, the area of square cell kidney disease is ri[e for new, basic, research.

Second, the field of square cell research is alluring because a new paradigm in the pathogenesis of square cell disease is emerging in which inflammation plays a prominent role. This new paradigm makes square cell kidney disease an ideal field of study for an apprenticeship in integrative physiology. In addition, the methods and expertise to study kidney inflammation in square cell disease are readily available at the University of Wenatchee, where mechanisms of acute kidney inflammation are a major research focus of the Renal Division.

Finally, the pathogenesis of organ dysfunction in square cell disease begins with vascular events, and the Acute Renal Syndrome frequently evolves in acute renal injury. Given my previous research focus -abnormalities of the renal

circulation in experimental models of acute renal injury- the decision to study square cell disease represents more of a redirection than a major change.

If awarded, the K08 will greatly facilitate my development as an independent physician-scientist. The proposed research requires that I am trained in several new areas and methodologies (see table below). Therefore, I plan on working closely with mentors and collaborators for the next several years until I have acquired the necessary skills and expertise. Although I take advantage of my mentors' and collaborators' expertise, my research focus is clearly distinct from theirs, as well well as from the bulk of square cell and renal researchers. Thus, the research and training plan outlined for this K08 award will very likely lead to an independent line of research in an area of specialized expertise.

C. CAREER DEVELOPMENT/TRAINING ACTIVITIES

The proposed research is interdisciplinary in nature, requiring expertise in physiology of the renal circulation, mechanisms of acute kidney inflammation, and cell-cell adhesion. Drs. Lou (primary mentor) and Gertrude (secondary mentor), and Terry (collaborator) are experts in these respective areas and are committed to providing training for the proposed research. In addition, Drs. Lou and Gertrude are strongly committed to integrating techniques used in their respective labs, and Dr. Terry has a long track record of collaboration with our Renal Division.

If awarded, this application will provide important training in cellular / cytokine physiology and cell-cell adhesion, two areas of research that are new to me. In addition, the proposed research will increase my versatility as a physiologist by introducing the use of transgenic mice, an ex vivo pump-perfused kidney model, and monoclonal antibodies. Training in these new fields of study and methodologies (see Table, below) is essential to my success as an integrative physiologist and square cell – kidney researcher.

As a primary mentor, Dr. Lou will be responsible for supervising progress of my entire research and training program including progress of all ongoing studies, review of manuscripts and grant applications, and career development. I will meet with him weekly throughout the training period. Dr. Lou will closely supervise the design, interpretation and troubleshooting of all experiments, with particular emphasis on isolated kidney experiments and physiological aspects of mouse experiments. New skills that will be learned in Dr. Lou's laboratory include development of the ex vivo rat kidney model and in vivo model of kidney injury in square cell mice.

As secondary mentor, Dr. Gertrude will be responsible for supervising training in cell/molecular biology including new methodologies, and will assist in design and interpretation of experiments related to mechanisms of kidney inflammation. I will meet with Dr. Gertrude at least monthly and more frequently when necessary.

As collaborator, Dr. Terry will have responsibility for supervising aspects of research evaluating adhesion pathways and using monoclonal antibodies. I will meet with her 1-2 times per month when the relevant experiments are being planed, performed, and interpreted. Although all planned training in cell/molecular biology will take place in Dr. Gertrude's laboratory, Dr. Terry will also provide training if experimental results suggest the need for different approaches. Based on anticipated problems (detailed in the research plan), this training may include use of flow cytometry, real-time polymerase chain reaction, and RNAse protection assays. Dr. Terry will provide me with the monoclonal antibodies for the adhesion experiments.

Dr. Dale will provide the transgenic-knockout mouse model of square cell disease for my studies. In addition, he will train me in how to properly design and interpret studies that use

Table. Training Value of Proposed Research

NEW FIELDS OF STUDY
Square Cell Disease*
Acute Kidney Inflammation*
Cell-cell Adhesion*
NEW METHODS/TECHNIQUES**
Small Animal Disease Models
Ex Vivo rat kidney model*
Kidney injury in transgenic mice*
Use of Monoclonal Antibodies*
ELISA technology*
Immunohistochemistry*
Trans-Bladder Ureter Catheterization*
Urinary Protein Quantification*
Urinary Cellular Analysis*
Myeloperoxidase Assay*

*indicates no previous experience
** Some additional techniques may be required

genetically altered mice. I will meet with him every 1-2 months when experiments with square cell mice are taking place.

Drs. Terry and Dale will also provide the helpful perspectives of accomplished physician-scientists in the field of hematology with interests in square cell disease. These perspectives will assist me in understanding how my work relates to other research in the field, and in anticipating future trends in square cell research.

A career advisory committee that I have had in place for the last three years will provide advice regarding my research progress, direction, and career development. Members of this committee include Dr. Lou, Dr. Gertrude, Dr. Eric Bates and Dr. Carmen Kidwell. Dr. Bates is an experienced renal physiologist who is Professor of Medicine and renal/critical care section head at the University of Wenatchee Medical Center. Dr. Kidwell is Professor of Medicine and renal/critical care section head at the Entiat River Cancer Research Center with research interests in the cell/molecular biology of acute renal injury.

Additional research training will occur through attendance of a weekly physiology study group, biweekly cell/molecular biology study group, weekly Renal Division research seminar, and monthly journal club. I will regularly present my work at the two study groups, periodically present my work at Dr. Gertrude's and Dr. Terry's lab meetings, and formally present my work twice yearly at the Renal Division Research Seminar. I will also attend seminars offered periodically by the School of Medicine on grant and manuscript writing and ethics in biomedical research.

Formal coursework at the University of Wenatchee will provide additional research training that will increase my sophistication in areas of cell/molecular biology relevant to square cell disease. Specifically, these courses will increase my understanding of cytokine physiology, ligand-receptor interactions, osmotic stress, and subcellular processes. The following courses have been selected:

Immunology 532. Advanced Immunology. Examines the molecular and cellular basis of immune function including antigen receptor structure, antigen presentation, and cytokines

Biology 401. Cell Biology. Reviews selected topics in molecular and cell biology with emphasis on understanding original experiments that describe cell functions.

Pharmacology 530. Pathways of Receptor Action. Focuses on molecular events related to ligan-receptor interactions and intracellular signaling.

Pharmacology 536. Transport Mechanisms in Health and Disease. Reviews biological active transport for proteins and ions, emphasizing their functions in fluid exchange dynamics, protein re-uptake, and the role of transport systems in disease processes and specific pharmacologic interventions.

Additional responsibilities during the period of this award include attending one-half day per week in the renal clinic, and one month per year on the inpatient wards. Inpatient responsibilities include renal/critical care consultation on hematology, oncology, and bone marrow transplant patients, including patients with square cell disease. In addition, I will be a small group instructor for the 4-week medical school renal physiology course (~9 hours/week). This leaves 80% of my time to focus exclusively on the proposed research.

SPONSOR'S STATEMENT

It is a real pleasure to provide my strongest possible support for Dr. Wayne Lawrence's K08 application. I have worked closely with him while caring for patients now as his mentor in the laboratory. He is fully committed to a career in medical research and I believe an ideal candidate for this award. Dr. Lawrence will acquire new tools and use innovative methods to gain important insights into causes of renal vaso-occlusion and the Acute Renal Syndrome in Square Cell Disease. Dr. Lawrence is beginning to differentiate himself from the traditional work in my laboratory. In doing so, he is establishing himself as an independent scientist. I believe we have constructed an exceptionally strong training program that will ensure his success as a productive physician-scientist.

Dr. Lawrence has all of the necessary qualities to succeed as an independent research-scientist. Foremost is the vitality and commitment he brings to the laboratory. His enthusiasm for his current projects and science in general, is obvious from the attitude with which he approaches his daily work. It is apparent that he really enjoys scientific research and is very excited about the new line of research that he has chosen. He is quite resourceful and knows when and where to seek assistance. He is adept in the animal laboratory and has become proficient with new investigative tools. I believe that as a research scientist, Dr. Lawrence is one of the best fellows in our program.

Dr. Lawrence demonstrates his potential for scientific research through this thoughtfully conceived and clearly articulated research application. Although Dr. Lawrence's initial studies explored the spatial distribution of renal perfusion in endotoxemia, he has become interested in the renal manifestations of square cell disease. This interest was inspired through work with Dr. Mike Goldwin at the NIH. Dr. Lawrence has developed this application through careful review of the literature, critical analysis of the data, and extensive conversations with other scientists. He has identified and formulated important questions. His hypotheses and experimental studies are carefully devised to specifically address these questions. Within this application, dr. Lawrence clearly presents results of his pilot studies and succinctly articulates his plans for future experiments.

The vast majority of work in vascular injury related to square cell disease has been performed in the systemic circulation. Dr. Lawrence will use this foundation of scientific work to explore vaso-occlusion and the role of adhesion molecules in the renal circulation. Because of significant differences between the two circulations, direct extrapolation to the renal circulation is not possible. Dr. Lawrence's work is guaranteed to provide new and important insights into vascular injury in the kidney secondary to square cell disease.

Summarized remainder of sponsor's statement:

The mentor describes how the candidate has shown ability to win an NRSA for his Post Doctoral work (ongoing) and a local grant for technical and laboratory support. He lauds his excellent writing and oral presentations kills. He indicates the time on the K08 will be well spent, and that the desire for a career in research is strong and sincere.

The training plan is outlined, and re-iterates many points in the candidate's statement, including protected time, new methods to learn, close interaction with senior scientists, lab space and technical help available, transgenic mouse is available for his use, career guidance for established scientists, and forums to polish presentation of his work. In addition, he will teach small classes, and serve limited time in the clinics; with 80% overall time protected for research.

The importance of the project is described, as is the overall approach and the list of collaborators who will aid the planning and analysis of the work as it progresses. A list of 5 other fellows the mentor has or is training is provided. The mentor clearly has time to spend with Dr. Lawrence.

The virtues of the co-mentor (Dr. Gertrude) are extolled, as are those of the main collaborator, Dr. Terry. The presence of CORE FACILITY support from a program project and a SCOR grant are described, along with other departmental support; The course work is defined as fitting clearly with the candidate's needs. The research integrity program is mentioned as being a well established year-long series of lectures. The candidate's ADVISORY COMMITTEE is mentioned here again.

The overall research environment, animal facilities, a second program project in a closely related area, and veterinarians and 4 animal technicians are mentioned who will be there to help Dr. Lawrence. A small animal lab has been designated for Dr. Lawrence to use. As in the candidate's statement, study groups and lab meetings are up and running and will be part of Dr. Lawrence's support system. Dr. Lawrence' publications are mentioned, with a paper ready for publication, and excellent prospects for an excellent rate of publication in the future.

The mentor, Dr. Lou, re-states his enthusiasm for the candidate's future as a productive scientist and eventual national leader in this field.

The mentor signs the letter,

Dr. McKinzie Lou is an associate professor.

LETTERHEAD OF THE SECONDARY SPONSOR'S INSTITUTION:
Sequim Medical Research Center
affiliated with the University of Wenatchee School of Medicine

From: Dr. Alice Gertrude
To: the K08 Review Committee
Re: Wayne S. Lawrence, MD (K08 Applicant)

SUMMARY OF THE LETTER'S CONTENTS

The co-sponsor states his strong qualification for co-mentoring, including familiarity with the candidate, and expertise in the area, and willingness to serve. She extols the virtues of the candidate as smart, enthusiastic, knowledgeable, and cooperative, as well as an outstanding teacher.

The letter tells about the research plan, and the importance of the work, as well as the world-renowned expertise of the mentor and collabotrators. The co-mentor emphasizes that the training plan and project were designed by the candidate himself, and will lead to a unique career niche that will put him at the forefront of workers in the kidney research field.

The research plan is re-iterated.

The strength of the environment is described.

A promise to provide strong support of the candidate is made; the strength of the mentor, the value of the program are re-emphasized; the studies are described as novel and important. Faith in the potential of the candidate is expressed.

Dr. Gertrude is Professor and vice chair of the Dept. of Medicine, University of Wenatchee, and Chief of Medicine at the Sequim Medical Research Center.

LETTERHEAD OF THE
UNIVERSITY OF WENATCHEE SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
Queets Research Center
DIVISION OF HEMATOLOGY

From: Dr. Annabel Terry, D. Sc., MD
To: Center for Scientific Review, NIH
Re: Dr. Wayne S. Lawrence

SUMMARY OF THE LETTER FROM THE COLLABORATOR

The collaborator, Dr. Terry, expresses her pleasure to collaborate with Dr. Lawrence; stating that she has carefully reviewed the research plan. She state how important and timely the research is. She describes how the known work in the square cell disease field is mainly on systemic circulation, not that of the kidney, where salt concentrations are likely to be important in explaining the pathophysiology of the disease there.

The candidate's superb training so far is described.

The collaborator describes his work on cell-cell adhesion in hematology, and how it fits with the candidate's planned studies, and how the techniques in her lab will be shared with the candidate, who will train there in their use. These will include: 'real time PCR', RNAse protection assay, ELISA, immunohistochemistry, confocal microscopy, flow cytometry. Dr. Terry will supply reagents for rodent studies, including anti CD99 monoclonal antibodies; in the future, if needed, she will teach the candidate cell / molecular biology approaches, e.g., DNA array or gene transfection.

Dr. Terry concludes by saying she has met with Dr. Lawrence several times and is impressed with his critical thinking and careful, reasoned approach to research problems. She observes that the candidate is excited about the research and has made outstanding progress; and should continue to do so on this important and clinically relevant project. She considers Dr. Lawrence an outstanding candidate for the K08 award.

Dr. Terry is Professor of Medicine,
Adjunct professor of pathology,
Head, Division of Hematology
University of Wenatchee

SUMMARY OF THE BIOGRAPHICAL SKETCH FOR DR. TERRY

Dr. Annabel Terry

BS Biology,	U. Chicago	1969
MD	Loyola Univ., Chicago	1973
Internship	UCSF	1973-4
Residency 1+2,	UCSF	1974-6
Fellowship	Univ. Wenatchee	1974-6

Dr. Terry has spent her professional career at the Univ. Wenatchee, progressing through the academic ranks from instructor to professor from 1978 to 1989. 1990 to current time is head of hematology.

Winner of many awards and honors

Certified in Internal Medicine, Hematology, and Medical Oncology

Member of several professional councils, serves on editorial boards of many publications

Has a program project, and is PI of a subproject on another; PI on subproject of a SCOR; has two R01 grants

List 30 relevant publications out of 200 in the last 8 years; most in Blood, Immunology; J. Exp Med; J. Biol. Chem; Am J. Pathol.; J. Cell Biol. Most as senior author

LETTERHEAD
UNIVERSITY OF WENATCHEE
DIVISION OF HEMATOLOGY

To: Wayne S. Lawrence, MD
From: Elmond Dale, MD

SUMMARY OF LETTER FROM DR. DALE

This letter from a collaborator shows "full" support for the candidate in carrying out his studies. Belief in the importance of the studies is expressed, and states that the pathology in the kidney for square cell disease is obscure but clinically important. Dr. Dale expresses confidence that important results will come form Dr. Lawrence using his (Dale's) mouse model, and it will benefit both labs.

Dr. Dale is impressed by the interdisciplinary team of mentors and collaborators Dr. Lawrence has assembled. Dr. Dale pledges support for helping with clinical and research aspects of square cell disease.

Dr. Dale is developing a gene therapy for square cell disease, and has a colony of mice devoted to this, which he will share with Dr. Lawrence, in addition to reviewing his research progress every one or two months. They will collaborate in using knockout and transgenic mice to study renal pathogenesis of square cell disease.

SUMMARIZED BIOGRAPHICAL SKETCH FOR DR. DALE:

Elmond Dale, MD

BS	Wayne State Univ	1982
MD	Iowa State Univ	1986
Internship	Duke	1986-7
Residency	Duke	1987-9
Fellowship	Univ. Wenatchee	1990-4
Clinical Tutor	Univ. Wenatchee	1992-present
Asst Prof.	Univ. Wenatchee	1994-present

Awards:
AOA, 1985
Hamilton Shipman Award, Duke, 1989, (excellence in clinical medicine)

Currently holds two R01 awards from the NIH

Certified in Internal Medicine, Medical Oncology, and Hematology

Total of 29 publications, lists 26, including two submitted for publication and a letter to NEJM, most are first author, most in Blood, with one or two in PNAS, Lancet, Hematology, J. Biol. Chem.,

5. Environment and Institutional Commitment to the Candidate

a. Description of Institutional Environment

SUMMARY OF THE DOCUMENT

This section is written by Dr. William Willett, the Chairman (and Professor) of the Univ. Wenatchee Department of Medicine. He notes the interdisciplinary nature of the work, notes the well-established local programs of research on the proposed fields, and that the roster of mentors and collaborators represent the required expertise.

He notes the Mentor's two R01 grants, and the similar strengths of the co-mentor and collaborators in winning grant support. He notes the weekly reviews of progress with the mentor, and monthly with the other collaborators, plus the regular lab meetings and departmental presentations. Course work is noted.

Formal progress towards independence will be reviewed twice yearly by the Departmental Research Training Committee, and he will meet twice yearly with his advisory committee. Dr. Lawrence will have access to consultants at the university for statistics, grant writing, and manuscript preparation, as well as the rich environment of scientific resources. He will have office and lab space, and a dedicated Apple G4 computer.

b. Institutional Commitment to Candidate's Research Career Development

SUMMARY OF THE DOCUMENT

Dr. Wm Willett continues: saying that the department will foster the career of Dr. Lawrence by providing adequate space, time, and resources for him to develop an independent research program. He will be promoted to Senior Fellow/Acting Instructor upon initiation of the project. The note states the Dr. Lawrence will be very competitive for an Assistant Professorship at the Univ. of Wenatchee at the mid-way point of this award. Clinical time is re-iterated as the 80% research, 20% clinical stated by the Candidate. Departmental supplementation of grant salary is pledged if the level is below that standard for Instructors at U of W. Dr. Lou's technical and supply money currently pay the costs of Dr. Lawrence's project, and will supplement the K08 support as needed.

Dr. Willett re-states enthusiasm for the candidate and the importance of the work, and potential for a productive research career as an independent physician-scientist.

SPECIFIC AIMS

Although renal complications are the leading cause of death in square cell disease, the kidney has rarely been the focus of basic research in this disorder. Current understanding of square cell pathophysiology derives from studies performed in vitro or in other organs. Because mechanisms of vaso-occlusion and inflammation in the kidney are likely to be different from those in other organs, there is a critical need for basic square cell research that focuses on the kidney. The proposed research will investigate mechanisms of renal vaso-occlusion and kidney inflammation in animal models of square cell disease. These experiments will improve understanding of square cell pathogenesis in the kidney and ultimately lead to new treatments for patients with renal complications of square cell disease.

HYPOTHESIS 1: Renal vaso-occlusion is caused by tubular hypertonia but not by mixed arterial hypertonia, and is enhanced by renal inflammation.

Specific Aim 1a: Measure vaso-occlusion over a range of tubular urine tonicities in isolated rat kidneys perfused with erythrocytes from patients with square cell disease (SQ RBCs).

Specific Aim 1b: Determine the effect of mixed arterial hypertonia on vaso-occlusion in isolated rat kidneys perfused with SQ RBCs.

Specific Aim 1c: Determine if kidney inflammation due to lipopolysaccharide (LPS) enhances renal vaso-occlusion, and if this effect is mediated by adhesion of SQ RBCs to the vascular wall via integrins c5b1 or c4b1.

HYPOTHESIS 2: Hypertonia-induced vaso-occlusion causes kidney inflammation and renal endothelial activation in a transgenic mouse model of square cell disease (“square cell mice”). Hypertonia-induced changes are in part mediated by effects of c4b1 on SQ RBC and leukocyte adhesion.

Specific Aim 2a: Measure leukocyte, cytokine, and chemokine responses and endothelial activation in kidneys of square cell and control mice that have been infused intravenously with hypertonic saline.

Specific Aim 2b: Determine if neutralizing antibodies to c4b1 attenuate hypertonia-induced renal inflammation in square cell mice.

HYPOTHESIS 3: Square cell mice exhibit an enhanced inflammatory response to LPS that depends on CD99 – mediated leukocyte recruitment.

Speficific Aim 3a: Measure leukocyte, cytokine, and chemokine responses in kidneys of square cell and control mice after intravenous infusion of emulsified LPS or saline.

Specific Aim 3b: Determine if neutralizing antibodies to CD99 attenuate LPS-induced kidney inflammation to a greater extent in square cell than control mice.

BACKGROUND AND SIGNIFICANCE

Why study Square Cell Disease in the Kidney?

Renal dysfunction commonly complicates square cell disease and is a major cause of morbidity and mortality. Acute Renal Syndrome is the leading cause of death in square cell disease and commonly leads to acute renal failure (58), while chronic uremia, filtration insufficiency, and renal vascular disease occur in 20-60% of adults with square cell disease (46, 54). Despite its clinical importance, the kidney has rarely been the focus of basic research in square cell disease. Current understanding of square cell pathophysiology derives from studies performed in other organs or in vitro. Because mechanisms of vaso-occlusion and inflammation in the kidney are likely to be different from those in other organs, there is a critical need for basic research on square cell disease that focuses on the kidney.

Square cell pathophysiology in the kidney is distinct from that in other organs for several reasons (summarized in Table 1). Square cell RBCs change shape, becoming cuboidal, and stack up, known as "brick-walling" when active transport falls, thus reducing glucose uptake to below critical levels for maintenance of discoid shape. Glucose metabolism is required for sub-membrane cytoskeletal function that maintains the RBC discoid shape. The molecular defect rests with ankyrin and band-4 (11, 47, 69, 86), which have lower affinity to actin and myosin type-2, requiring higher than normal energy expenditure to maintain shape, thus the susceptibility to the extremes of the renal micro-environment. Wild-type RBCs do not undergo this transition. In contrast to most organs, RBCs enter the kidney and encounter widely variable viscosities and tonicities intravascularly and their capillary endothelium must function under wide variations in extracellular fluid tonicity. Consequently, square cell disease manifests most strongly in the kidney through creation of micro-infarcts which accumulate irreversibly over years in these patients.

The micro-environmental effects are strongest in the renal medulla, where counter-current exchange produces a hypertonic extracellular fluid, the driving force for creation of concentrated urine. Upon leaving the renal medulla, dilution returns the tonicity to isotonic, and the SQ RBCs resume normal active transport and regain their normal shape. Brick-walling is also present in the renal cortex, where the factor influencing disc-square-transition is the removal of plasma from the blood at the glomerulus. This increases the viscosity of blood, and induces stacking of the RBCs, which for SQ RBC induces competition for limited amounts of glucose and thus induces a relative depletion of available glucose, and loss of maintenance of adequate intra-RBC energy source to maintain discoid shape, resulting in brick-walling of SQ RBCs in the vase recta of the proximal tubules, in the renal cortex. Again, this reverses as fluid follows the resorbtion of protein and ions by the proximal tubules, and intercellular fluid follows into the vasa recta, increasing plasma volume and reducing blood viscosity.

Table 1. Differences between kidney and other organ affecting square cell pathophysiology.

DIFFERENCES AFFECTING VASO-OCCLUSION	**OTHER ORGANS**	**KIDNEY**
Tonicity of blood in organ	unchanging	Hypertonic in medulla
Viscosity of blood in capillary transit	unchanging	Hyperviscous in renal cortex
Affects on trans-RBC solute exchange in transit	unchanging	Impaired in renal medulla
Square cell formation	Slow in iso-osmotic condition	Rapid in hypertonic condition
Hemodynamics	Reflect systemic pressure	Variable under local and systemic control at glomerulus
DIFFERENCES AFFECTING INFLAMMATION		
Mechanisms /site of neutrophil recruitment	Selectin-dependant CD99 dependent Post-capillary venular endothelium	Selectin-independant May be CD99 independent Vasa-recta capillary endothelium
Role of glomerular mesangial cell	none	important
Role of tonicity of extracellular fluid	Isotonic-no effect	Hypertonic-inhibitory (in medulla)
Affect of vaso-occlusion	Ischemia, waste accumulation	Ischemia, cessation of resorbtion
Susceptibility to ischemia / reperfusion injury	high	higher

Mechanisms of inflammation and tissue injury are likely different in the kidney, for some of the same reasons, including the lower viscosity stacking blood neutrophils amongst the RBCs, and the renal medullary hypertonicity affecting plasma membrane active transport as well as affecting receptor-ligand interactions for inflammatory signaling. This is most important for CD99 dependant neutrophil adhesion, which is singularly enhanced in the hypertonic conditions of the renal medulla.

Physiological Determinants of Vaso-occlusion in the Kidney
Research on renal vaso-occlusion is limited to two studies that suggest severe medullary hypertonia causes sequestration of SQ RBCs in the kidney (3, 17). These studies did not adequately assess effects of modest tubular hypertonia and no study has evaluated the importance of mixed arterial hypertonia or inflammation to renal vaso-occlusion. In **Specific Aim 1**, we adapt the isolated rat kidney model used in these original studies (3, 17) to determine effects of tubular hypertonia, and mixed arteriolar hypertonia and renal inflammation on kidney micro vaso-occlusion.

Mixed arteriolar hypertonia is the combination of reduced plasma volume (and higher viscosity) with hypertonic plasma moving up from the renal medulla,which combine at the cortico-medullary junction, the site of most renal vaso-occlusion and micro-infarcts in square cell disease. Mixed arteriolar hypertonia may contribute to vaso-occlusion because 10-30% of SQ RBCs in mixed renal blood are in the square (cuboidal) form and can be observed histologically as 'brick-walls.' The squared cells disassociate upon leaving the junction, but stasis results in microinfarcts. **Specific Aim 1** will test this by using isolated rat kidneys in which the tonicity and plasma volume can be controlled, and using human SQ RBCs to assess trapping. Trapping will be assessed regionally. **Specific Aim 1a** we will control tonicity by infusing hypertonic saline into the ureter of the isolated rat kidney. In **Specific Aim 1b**, the mixed arteriolar hypertonia will be modeled by infusing human SQ RBCs at various hematocrits along with controlling tonicity by infusing hypertonic saline into the ureter of the isolated rat kidney.

Effect of Inflammation and SQ RBC Adhesion on Vaso-occlusion
Activated endothelium, and injured endothelium allows exposure of the underlying capillary basement membrane, and its sub-endothelial matrix proteins, and increases adhesion of SQ RBCs as well as of platelets and inflammatory cells, especially neutrophils (20, 30, 40). All these potentiate vaso-occlusion. Because inflammatory mediators are frequently elevated in SQ patients (8, 15, 31, 52, 55), inflammation may create a pro-adhesive state that causes occult microvascular occlusion or clinical events such as vaso-occlusive crisis or Acute Renal Syndrome.

A number of studies have shown that inflammation increases SQ RBC adhesion (5, 30, 34, 40) but few have provided direct evidence linking inflammation to vaso-occlusion. In the most convincing study to demonstrate this link (30), platelet activating factor increased SQ RBC-endothelial adhesion and vaso-occlusion in the artificially perfused trat mesentery, and blockade of the pro-adhesive integrin c5b3 attenuated these events.

Although numerous pathways mediate adhesion of SQ RBCs (16), the integrin c4b1 on young SQ RBCs and the integrin c5b3 on endothelium appear to be the most important in the systemic circulation (16,32, 51). C4b1 mediates SQ RBC adhesion by binding to vasculaer cell adhesion molecule-1 (VCAM-1) on activated endothelial cells or fibronectin in the extracellular matrix. C5b3 is expressed to a greater extent in kidney than in other organs (47) and causes adhesion of SQ RBDCs by using vonWillebrand Factor (vWF) as a bridging molecule. Because these pathways have primarily been studied using in vitro preparations, their biologic relevance needs to be confirmed in intact organs or organisms. Furthermore, the relevance of these and other adhesion pathways in the kidney is unknown.

In **Specific Aim 1c**, we will test the hypothesis that systemic inflammation increases vaso-occlusion in the kidney by producing a pro-adhesive state mediated by integrins c4b1 and c5b3. I will evaluate kidney inflammation due to LPS because LPS activates endothelium (11, 27, 43, 56) and circulating LPS complicates square cell disease (8, 36, 55) due to sepsis, translocation of bacterial products from ischemic gut, or reticulo-endothelial dysfunction (55).

Mechanisms of Kidney Inflammation in Square Cell Disease

Recent studies suggest that square cell disease is exacerbated by inflammatory conditions, and contribute to organ damage. Neutrophils and monocytes in SQ patients are activated. Leukocyte counts are increased, especially in Acute Renal Syndrome. The role of inflammation in SQ disease is as yet unknown.

In **specific aims 2 and 3**, we propose experiments that will characterize the magnitude and mechanisms of the inflammatory response in kidneys of transgenic and knockout mice that express human ankyrin and band-4 of the RBC plasma membrane (cytoskeletal components whose defects are associated with the disc to cuboidal shape change), thus, SQ mice.

In **specific aim 2**, we propose to test that 'brick-walling' and capillary adhesion of SQ RBC contribute to endothelial activation and inflammation in the kidney. The activation state of kidney endothelium is unknown in SQ disease. In **specific aim 2a**, we test the role of vascular hypertonicity in activation of inflammatory mediators in SQ mice. In **specific aim 2b** we test the role of c4b1 integrin in SQ RBC adhesion and induction of inflammation in SQ mice. The test will be performed by infusion before saline challenge with blocking antibodies to c4b1. This experiment is especially promising because both SQ RBC adhesion and inflammatory cell adhesion rely on this ligand. The therapeutic consequences are obvious.

The pathogenesis of Acute Renal Syndrome is poorly understood. Although infection and fat/marrow embolism may precipitate Acute Renal Syndrome, the majority of cases are unexplained (58). We hypothesize that square cell disease is associated with enhanced inflammatory response in the kidney. By amplifying mild inflammatory insults that are trivial in normal hosts, an enhanced inflammatory response could explain the increased frequency of renal failure and kidney injury in patients with square cell disease. In **specific aim 3a** we test the hypothesis that leukocyte, cytokine, and chemokine responses in kidneys of square cell and control mice are enhanced after intravenous infusion of emulsified LPS as compared to saline control. In **specific aim 3b** we will determine if enhanced inflammation in square cell disease is mediated by leukocyte adhesion using blocking antibodies to CD99. CD99 is the b2-integrin subunit of Moc-1 (CD86b/CD99) and lymphocyte-associated function antigen-13 (CD46/CD86), the two integrins most responsible for neutrophil binding to activated endothelium (59).

Summary and Clinical Significance

Studies performed in vitro and in other organs have given important insights into the pathophysiology of square cell disease, but have not yet defined the important pathophysiology in the kidney. The studies we propose will attack the problem directly using sensitive and specific techniques. These studies will lay the experimental foundation for understanding square cell disease crises in the kidney. The importance of these studies to the affected population cannot be exaggerated.

PRELIMINARY STUDIES

This is a synopsis of the presented studies

The application presents preliminary data supporting **specific aim 1a**, with an experiment in isolated rate kidney perfused vascularly with human SQ or control blood, and infused via the ureters with saline varied from isotonic to hypertonic and reversed back to isotonic. Under constant flow rate, perfusion pressure (resistance to perfusion) increased with increasing tonicity, and was reversed when tonicity returned to isotonic (n=4).

Specific aim 1b was supported by an experiment wherein the hematocrit of the human blood infused was raised to mimic extraction of plasma by glomerular filtration. Saline was infused at varying tonicity at the ureter. Increases in perfusion pressure at constant flow were higher as hematocrit was raised, and there was a synergistic increase in pressure upon raising the urine tonicity (n=1).

Specific aim 1c was supported by data suggesting that LPS increases vaso-occlusion in the hypertonic-urine infused kidney. Rats were infused with emulsified LPS, kidneys isolated, canulated, and infused arterially with SQ RBCs and infused retrogradely via the ureters with saline of increasing tonicity. Perfusion pressure increased more rapidly that in non-LPS pre-treated rat kidneys (n=4).

Specific Aim 2a was supported by an experiment wherein square cell (SQ) and control mice were infused arterially with hypertonic saline to induce hypertonicity in the renal parenchyma. After 24 hour infusion, blood, urine, and kidney homogenate were assayed for mediators of inflammation. Chemokines CK, IL8-B, and KE/CMP-4, were elevated 6 to 8 fold in homogenized kidney, and 3 to 5 fold in blood only in the SQ mice after the treatment (n=1). It was noted that KE/CMP-4 was elevated 2 fold in untreated SQ mice when compared to controls (n=1). KC and KE/CMP-1 are mouse homologs of inflammatory mediators in humans that chemo-attract and activate neutrophils. In addition, histology showed micro thrombi and 'brick-wall' stacks of cuboidal RBCs, mainly but not only, at the cortico-medullary junction of the kidney; this was observed only in the SQ mice (n=1).

Specific Aim 2b was not mentioned in the preliminary studies.

Specific Aim 3a was supported by an experiment in which LPS was infused for 30 minutes, then animals sacrificed 4 hours later. In SQ mice, urine neutrophil counts, and kidney homogenate acute inflammatory cytokines IL-8 and TNF-d, and chemokines Ke/CMP-4 and CK were all elevated compared to wild-type mice (n=1). Oddly enough, however, the increases in the markers of acute inflammation were elevated 2 fold higher in heterozygous SQ mice than in homozygous SQ mice. The chemokines were elevated to the same degree in the hetero- vs. homo-zygous SQ mice.

Specific Aim 3b was not mentioned in the preliminary studies.

RESEARCH DESIGN AND METHODS

THESE ARE COMMENTS GATHERED FROM THE SUMMARY STATEMENT

REVIEWER 1
Design:

The studies are designed to investigate the mechanisms of renal vaso-occlusion triggered in the isolated perfused rat kidney by hypertonia and in square cell mice by tubular hypertonia or emulsified endotoxin. The overall hypothesis pursued is that both tubular (renal medullary) hypertonia and inflammation lead to vaso-occlusion and also that vaso-occlusion, per se, causes kidney inflammation and renal endothelial cell activation. The applicant will use the isolated pump perfused rat kidney system to study vascular occlusion triggered by tubular hypertonia and by changes in the mixed arterial hypertonia modeled by varying the hematocrit in the perfused blood. This methodology is relatively straightforward, the applicant did show in preliminary experiments that tubular hypertonia causes a rapid rise in the renal arterial pressure when the perfusate contained square cell red blood cells and that the increase in renal arterial pressure was even greater when the isolated kidneys were derived from rats pretreated with LPS. In addition to a histological assessment of the degree of vaso-occlusion, the applicant wishes to use 51Cr RBCs and measure the remaining radioactivity following flushing of the kidney circulation. As stated, no pilot experiments are provided for this technique and it is unclear how sensitive this method really is and whether this method can detect differences in cell retention following challenge of the kidney preparation with different degrees of hypertonia.

The applicant wishes to differentiate between the effects of tubular hypertonia and mixed arterial hypertonia; however, the experiments are not well described in detail to allow their assessment. It is not clear how the mixed arterial hypertonia will be controlled independently from the tubular hypertonia. The remainder of the experiments will be conducted using square cell mice, which are being made available to the investigator by Dr. Elmond Dale. In the pilot experiments, the applicant did show that 24 hour exposure of square cell mice to hypertonia (IV hypertonic saline, via catheter) causes histologically an accumulation of thrombi in post glomerular venules and vasa recta.

Apparently, the site of vaso-occlusion is different in these mice when compared with the hypertonic rat kidney. Experiments are described where the applicant wishes to examine the combination of hypertonia and LPS to assess the degree of vaso-occlusion by square cell RBCs. Again, it is not clear whether the methodology applied will be sensitive enough to distinguish between different grades of occlusion. The experimental use of anti-human monoclonal antibodies directed against c5B1 however are quite exciting. The applicant also plans to use neutralizing antibodies directed against CD99 in order to attenuate the LPS kidney inflammation - the principle readout in these experiments will be the measurement of kidney tissue homogenate- and urine cytokines and chemokines.

Overall, this series of experiments will provide new insights in the mechanism of renal vaso-occlusion in the context of square cell disease. Some of the initial studies are justifiably descriptive, but functional assessment using antibody treatment strategies are also provided. An important aspect of this proposal is the aspect that neutrophil adhesion may be an important factor in the pathobiology of the square cell renal syndrome.

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RESEARCH DESIGN AND METHODS

THESE ARE COMMENTS GATHERED FROM THE SUMMARY STATEMENT

REVIEWER 2

Research Plan:

The applicant proposes to use isolated rat kidneys and transgenic square cell mice to explore mechanisms of square-related renal vaso-occlusion and inflammation. Aims will investigate the role of tubular and mixed arterial hypertonia, inflammation and erythrocyte-endothelial adhesion.

Aim 1A and B test the idea that tubular urine hypertonicity and mixed arterial hypertonia and kidney inflammation enhance square-associated vaso-occlusion. This seems feasible, and probably worthwhile, but will contribute relatively little important new mechanistic information. The findings will, for example, fail to distinguish effects of cubing and 'brick-walling' vs adherence with respect to vaso-occlusion. The studies will also be unable to distinguish the influence of pre- vs intra-renal delay. The arteriolar and capillary bed of the normal kidney is only partially perfused at rest and this provides a potential dwell time in the pre-capillary circulation which could enhance cubing, brick-walling, or adherence. Reasons for including very severe conditions for delay and hypertonia are unclear and the findings will be of uncertain relevance. Given that hypertonia may augment RBC-endothelial adherence (ref 51) studies of the effect of anti-adherence site agents would be a useful addition to these studies. **Aim 1C** determines the extent of enhancement of vaso-occlusion induced by endotoxin (LPS). Given the published findings of square RBC adherence to cultures of renal and systemic endothelium, and the defined role of integrins and VCAMs the findings seem predictable. However, direct demonstration that endotoxin enhances vaso-occlusion in the perfused kidney will have some value. Given the likely role of VCAM-1 in square RBC-endothelial adherence (Swerlick, Blood 82:1891-99,1993) antibodies to block this adherence site might be a useful addition to these protocols..

Aim 2 will determine whether hypertonic vaso-occlusion induces kidney inflammation and endothelial activation in square mice and tests the role of c5B1 integrin in leukocyte adhesion. In these studies it is unclear whether all phases of kidney harvesting and processing will be in hypertonic conditions in order to avoid the demonstrated square vascular injury reported with reperfusion (ref 29). As noted earlier, anti-VCAM-1 antibodies might add useful information to the studies in **aim 2B**.

Aim 3 will determine whether inflammatory responses to infused endotoxin are increased in square mice and the role of CD99 in leukocyte recruitment. The rationale for administration of LPS by infusion is clear and seems to fit well with the generally held and applicant's stated view that endotoxin in square cell disease may more likely reach the kidney by the vascular rather than by the urinary route. Overall strengths of the proposed research are the importance of work in the clinically important, but under-investigated area of square-related and inflammatory influences in the kidney. The applicant's preliminary data and the expertise of the mentors and consultants point to the likely feasibility of the proposed studies. Weaknesses include the problem that a substantial proportion of the work will likely merely confirm that characteristics of square cell - endothelial interactions observed in cultured and systemic endothelium also occur in the kidney. Further, the important aspect of the square cell-inflammation linkage leaves unaddressed the sequence of the relationship. Does initial square cell-endothelial interaction promote secondary leukocyte adherence and inflammation, or does initial inflammation induce enhanced subsequent square RBC adherence? These could be addressed by sequential exposure studies to determine whether kidney perfusion with square cells followed by subsequent perfusion with neutrophils or platelets induces adherence of these elements. If so, through what mechanisms? What are the potential roles of filtration effects and decreased active transport activity? A shift in focus to a more mechanistically driven research plan could strengthen the application.

Projected 5 year Time Line for Experiments

Aim	Year 1	2	3	4	5
1a	xxxxxxxxxxxxxxxxxxxxx				
1b		xxxxxxxxxxxxxxxxxxxxxx			
1c				xxxxxxxxxxxxxxxxx	
2a-b	xxxxxxxxxxxxxxxxxxxxxx				
3a-b			xxxxxxxxxxxxxxxxxxxxxxx		

APPLICATION'S CRITIQUE OF THE EXPERIMENTAL APPROACHES

This is a synopsis of the text in the application

For all experiments, ANTICIPATED PROBLEMS AND ALTERNATIVE APPROACHES are addressed. Detailed and reasonable INTERPRETATIONS of each set of experiments are included.

The applicant notes that the vaso-occlusion (RBC trapping) studies are indirect, but hope the combination of approaches will be convincing that vaso-occlusion is being measured. FUTURE PLANS call for intravital microscopy to be learned from Dr. Wilson Stilz, of the University of Nebraska, who has been doing this in kidney for years in collaboration with Dr. Lou's group.

Increases in renal artery pressure during RBC infusions might be to small to be reliable, so pulsatile flow or higher hematocrit might be tried to overcome this.

The perfusates are all un-physiological with artificial plasma, simple saline, manipulated RBCs, and high flow rates. These problems are acknowledged and promises are made to address them 'if necessary'; rat plasma might be used, for example, or other rat-derived blood components.

Effectiveness of blocking antibodies will be checked if null hypotheses are reached in those experiments, although it is stated that cross-reactivity has been good between human and rat cytokines.

Chemokine responses have been measured at single time point, but they vary with time, likely requiring multiple time point sampling.

Gene expression is not included as an experimental technique in this proposal, but could be used (RNAse protection assay) to quantify chemokine responses.

Immunohistochemistry is not quantitative,, but real-time PCR is to be learned in the Terry lab, and could be used.

Numbers of experimental animals in each experiment are variously planned to be 12, 20, or 24 per experimental arm, with no mention of power calculations to assure statistical validity of these choices; however, ANOVA is mentioned as a test for all experiments.

(text now returns to that of the application)

HUMAN SUBJECTS

Human volunteers with and without square cell disease will donate 15-20 ml of venous blood after written informed consent. Volunteers with square cell disease who take medicine for the condition, or have had a blood transfusion within 3 months, or are experiencing a vaso-occlusive crisis or Acute Renal Syndrome are excluded. Any volunteer who reports being positive for HIV, hepatitis C, or has square cell trait will be excluded. Because of the demographics of square cell disease, volunteers will be from all races. Equal numbers of men and women will be recruited. We are currently approved to enroll volunteers age 18 and older. A revised human subjects form is currently pending so that we may include volunteer as young as age 12. Volunteers will be recruited by flyers posted in the University of Wenatchee Medical Center and affiliated hospitals, and the hematology clinics at the Sequim Medical Research Center and at the Queets Research Center. Participation in the study is voluntary and anonymous. Risks of a venous blood draw are minimal. Volunteers are currently compensated $10 per donation. When additional funding is available, patients with square cell disease will be compensated $40 per donation. We estimate that 37 volunteers with square cell disease and 25 volunteers without square cell disease will be enrolled evenly over the 5 year period.

VERTEBRATE ANIMALS

This proposal requires 136 Sprague-Dawley rats (~300 g), 80 square cell mice, 80 heterozygous mice, and 80 C57B1/6 wildtypr mice over a 5 year period. Roughly one fifth of these totals will be required each year. These numbers are estimates based on preliminary data and work of previous investigators using the isolated rat kidney model (3, 17) and vascularly infused emulsified LPS model (48).

Number of Animals over Time

Aim	Year 1	2	3	4	5
1a	xxxx 20 rats xxxxxxx 24 rats				
1b		xxxx 12 rats xxxxxxxxx 32 rats xxxx			
1c				xxxx 24 rats xx xx 24 rats xxx	
2a	xxxxx 60 mice xxxxx				
2b		xxxxx 60 mice xxxxx			
3a			xxxxx 60 mice xxxxx		
3b				xxxxx 60 mice xxxxxxx	

We use rats in specific aim 1 because no other ex vivo kidney model of square cell disease exists, and rats kidneys are frequently pump-perfused. Rats will undergo terminal surgery in which their kidneys are isolated after a surgical stage of anesthesia has been achieved with ketamine (60 mg/kg IP) and xylazine (7 mg/kg IP). A toe pinch will be used to ensure adequate anesthesia prior to surgery. 24 rats will receive LPS (Ecoli serotype 0111:B4) at an intermediate dose (6 mg/kg IV) and will be observed for 24 hours prior to surgery as above.

Specific aims 2 and 3 will use transgenic-knockout mice that express the mutant RBC cytoskeletal proteins ankyrin and Band-4 responsible for Square cell disease. We use transgenic square cell mice because they are the only in vivo model of square cell disease. Mice that exclusively express the mutant human cytoskeletal proteins ankyrin and Band-4 (homozygous mice), mice that express normal murine and mutant human ankyrin and Band-4, and C57B1/6 wild-type mice will each be used in specific aims 2 and 3. Because heterozygous mice manifest mild abnormalities of square cell disease, wild-type mice are required as a second control group.

Mice will be observed for 24 hours during delivery of hypertonic saline through a non-mobility impairing IV catheter (mice caged separately) (specific aim 2) or observed for 3.5 hours following a 30 minute infusion of

emulsified LPS (specific aim 3). One quarter of the mice in aims 2 and 3 will receive intraperitoneal injections of monoclonal or control antibodies in an effort to block the inflammatory response to hypertonia or LPS. At the end of the observation period, mice will be given a lethal overdose of pentobarbital and their kidneys harvested.

All animals will be observed frequently during experiments. Animals experiencing undu stress (e.g., inability to remain upright, severe lethargy, cyanosis) will be euthanized with a lethal overdose of pentobarbital. Animals will be housed in University of Wenatchee vivariums within the medical center complex. Vivariums are supervised by the Department of Comparitive Medicine and are AAALAC- accredited. Facilities and animal husbandry are in compliance with the standards outlined in the NIH publication "Guide for the Care and Use of Laboratory Animals." The colony of transgenic-knockout square-cell mice will bred in specific-pathogen-free environment in the University Medical Center complex by my collaborator Dr. Elmond Dale. The methods of euthanasia described above are consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

References

(Not Included in this text)

Letters of Recommendation for LAWRENCE

Letter 1

Dr. Elliot Edsel Writes his enthusiastic support for Dr. Lawrence, stating his belief in the candidate's potential for developing into an extremely productive independent investigator. He states the clear commitment to a career doing translational research relevant to clinical problems. He mentions the excellent mentoring and collaborators in a strong research environment. The importance of the problem behind the research project's aims is stressed.

Dr. Edsel is a Professor of Medicine
Head, Div. Renal and Critical Care medicine
Endowed Chair, Renal Disease Research
PI of a SCOR in Acute Renal Disease, Univ. Wenatchee

Letter 2

Dr. Eric Bates (member of the candidate's advisory committee) writes his strong support, citing first that he reviewed Dr. Lawrence's first manuscript, which he still cites in his work. Dr. Bates states his confidence that the candidate has exceptional promise, and mentions that the candidate's second and third published papers are important and provide new information about renal blood flow and inflammation. He now has confidence that the currently proposed studies of square cell disease have a high probability of providing new mechanistic insights into square cell syndrome. He remarks of Dr. Lawrence's hard work, insightfulness, enthusiasm and drive. He states his belief that the candidate will have an important biomedical career.

Dr. Bates is Professor of Medicine, Physiology and Biophysics
And Renal and Critical Care Medicine

Letter 3

Dr. Carmen Kidwell (member of the candidate's advisory committee) offers her strongest support and recommendation for Dr. Lawrence. She says she has known the candidate since 1997 and has served on his Career Advisory Committee and has observed the development of his research and academic career plans. She observes the candidate has an intellectual curiosity that is driving him to do research, and has great enthusiasm for the work. She says his work thus far is original and uses novel methods; his current proposal is integrative of physiology and cell biology, and has proposed a thoughtful and significant concept of combined inflammation and vaso-occlusion in renal complications of square cell disease. She closes with saying Dr. Lawrence has dedication and intellect, and is committed to a research career. She gives him her strongest support.
Dr. Carmen Kidwell is Associate Member, Div. Renal and Critical Care
Entiat River Cancer Research Center
Professor of Medicine, Univ. Wenatchee