6-24-85

E.B. Hancock, CAPT, DC, USN, (Code 408)
Naval Medical Research and Development Command
Naval Medical Command
National Capital Region
Bethesda, MD 20814-5044

RE: Annual Letter Report
ONR Contract #N00014-84-K-0562
"Pharmacology of Periodontal Disease"

Dear Capt. Hancock:

Enclosed is the annual report for the first year of funding of this ONR contract. If you require additional information or clarification please do not hesitate to contact me. Many thanks for your continued support and interest in this project.

Sincerely,

Steven F. Hoff, Ph.D.
6-20-85

E.B. Hancock, CAPT, DC, USN, (Code 408)
Naval Medical Research and Development Command
Naval Medical Command
National Capital Region
Bethesda, MD 20814-5044

Annual Letter Report
ONR Contract #N00014-84-K-0562
"Pharmacology of Periodontal Disease"
Steven F. Hoff, Ph.D. (Principal Investigator)

A. Progress Report:

The specific aim of this contract is to identify antibacterial and anti-inflammatory agents, used individually or in combination, which are appropriate for preventing the exacerbation of acute periodontal disease under field conditions, and our first year milestone involves the establishment of anatomical and biochemical approaches for the quantitative evaluation of these drugs. This milestone has essentially been completed, though our methodological approach has been changed. We originally proposed to use the rice rat model as a system to evaluate the effects of the various drugs on periodontal disease. However, after several discussions with the research group at the Naval Dental Research Institute (Great Lakes Naval Base) and our own re-evaluation of the current literature and the specific aims of this study, we decided to begin our studies on isolated human polymorphonuclear leukocytes (PMN). There are several reasons:

1. The rice rat model does not accurately parallel the human disease state.

2. This model of periodontal disease involves a very complex tissue, containing many different cell types, which appears to be inappropriate for a very basic evaluation of drug effects and efficacy.

3. In humans, the PMN appears to be the primary cell type responding to the bacterial invasion and plaque-accumulation associated with periodontal disease. In turn they may cause subsequent progress to a chronic inflammatory state with destruction of supporting periodontal tissues.

4. Because of the PMNs importance in the periodontal inflammatory process, we can establish our drug evaluation assays on a very simplified biological model, which can be examined under unstimulated and stimulated conditions in vitro, thereby reducing our need for research animals and perhaps increasing our ability to clearly accomplish our goals.

5. Also, this in vitro system will allow us to reconstitute the tissue inflammatory process by co-culturing of two or more known cell types. Thus we will be able to carefully analyze the cell-cell interactions and drug effects under controlled normal and stimulated (ie. inflammed) conditions.
In order to begin these studies, we applied to the Human Subjects Protection Committee/Internal Review Board (IRB) for permission to draw and use human blood samples from normal, healthy volunteers. Permission was given and a copy of that letter and our consent form are enclosed for your records (Appendix I).

During this funding period we have established several methods to evaluate drug effects on the structure and function of the PMN. These include:

1. **PHAGOCYTOSIS ASSAY**
   
   This assay examines the phagocytic capacity of PMNs under various conditions, including drug treatment. Using \(^{3}H\)-Staphylococcus aureus, we can quantitatively monitor cell attachment, endocytosis and bacterial cell killing by the PMNs. In addition we can take PMN samples during this assay procedure to be used for morphological assessment of the phagocytic process. This is being done with electron microscopy and computer assisted morphometric analysis of bacterial uptake and organelle redistribution. Also where a drug affects phagocytosis, we are using immunocytochemistry, with monoclonal antibodies to cytoskeletal proteins, to examine the breakdown or restructuring of the cellular filamentous and microtubular networks. The computerized image analyzer is capable of quantitating the fluorescent antibody distribution within the cells.

2. **DEGRANULATION ASSAY**
   
   During an inflammatory response, PMNs are stimulated to release several types of specific granules, which contain different hydrolytic enzymes and other proteins. Under stimulated conditions we are assaying for (a) lysozyme, (b) beta-glucuronidase and (c) lactoferrin. In this way we are able to differentiate the types of granules released under various conditions, including drug treatment. The drugs of interest may block degranulation or differentially affect the specific granule release. Our biochemical assays are being correlated with quantitative electron microscopic morphometric studies to examine total granule release. In addition, differential release of specific granules is being quantitatively assessed by histochemically staining one type of granule for myeloperoxidase.

3. **CHEMOTAXIS ASSAY**
   
   Another very important aspect of the inflammatory process is the ability of the PMNs to migrate to the diseased site. The effects of various anti-inflammatory and antimicrobial drugs are being studied using an assay to monitor cell motility and chemotaxis (ie. the ability to migrate toward a stimulating chemical). PMNs are placed in the central well of an agarose-filled petri dish and allowed to migrate between the agarose layer and the bottom of the dish. One of five wells surrounding the cells has a solution of FMLP (N-formyl-methionyl-leucyl-phenylalanine), which is a potent chemotactic stimulant. PMNs can be incubated in the presence or absence of various drugs, and after two hours the cells are quickly fixed and stained. Two parameters of chemotaxis, distance traveled and area of cell migration, can be determined on the computerized image analyzer. In addition, various samples can be analyzed.
morphometrically by quantitative electron microscopy for organelle distribution and cytoskeletal arrangement.

The above sets of anatomical and biochemical assays provide very sensitive methods of evaluating drug effects on the response of human PMNs to normal and stimulated conditions. During June and July, 1985 we will begin our analysis of several non-steroidal anti-inflammatory and antimicrobial agents in these assay systems. The compounds presently available to use are listed below.

NON-STEROIDAL ANTI-INFLAMMATORY AGENTS:
- Indomethacin
- Meclofenamate Sodium
- Sulindac
- Fenoprofen Sodium
- Ibuprofen
- Naproxen Sodium
- Tolmetin Sodium

ANTIMICROBIAL AGENTS:
- Clindamycin Phosphate
- Chlorhexidine Gluconate
- Tetracycline HCl
- Doxycycline Hyclate
- Amoxicillin Trihydrate
- Potassium Clavulanate
  (a beta-lactamase inhibitor)

B. Work Plan for Second Year:

Our work plan for the coming year includes the critical evaluation of many of the compounds listed above. First, each drug will be analyzed alone for its effects on human PMNs in the above assays. In addition we will begin our studies of the drug effects on cultured human periodontal fibroblasts (American Type Culture Collection). These cells are important to the normal structure and function of periodontal tissue and are known to be adversely affected by inflammatory processes. Using anatomical and biochemical procedures we will analyze their plating efficiency and ability to produce collagen in the presence or absence of the various drugs. If time permits, we will begin our studies on co-cultured human PMNs with the fibroblasts to examine the cell-cell interactions under stimulated conditions and the associated drug effects.

Second Year Milestone:
We will complete our evaluation of the various drugs used singly in the human PMN assays and if possible the cultured fibroblast assays also. From the results of these studies we will be able to identify non-steroidal anti-inflammatory and antimicrobial agents, which will be evaluated in the third year of this contract for their combined effects in our assay systems. It is expected that the drug combinations will provide a new and useful approach to the prevention of chronic periodontal disease.
C. Budget for Year Two:

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Equipment List and Justification:

Tissue Culture Equipment:
- Laminar Flow Hood: $7500
- CO₂ Incubator: 4500
- Nikon Inverted Microscope: 11,000

Total: 23,000

We will use the tissue culture facility to accomplish our second and third milestones, which include the use of cultured human periodontal fibroblasts and human PMNs co-cultured with the fibroblasts for our studies on anti-inflammatory and antimicrobial drugs. In addition, this equipment will enable us to conduct the proposed drug distribution studies during the third year of our contract.

Presently, tissue culture facilities are not available within the Pharmacology Department, and facilities within Microbiology and Biochemistry are heavily utilized and cannot provide the space or time necessary for our experiments.

Second year budget comments:

Minor adjustments have been made in several budget categories. The sets of anatomical and biochemical assays we have selected have proven very labor intensive, and I have adjusted the Personnel category to maintain the current level of staffing during the second year. The Supplies category has been slightly decreased because of the staffing needs; however, this will not jeopardize our ability to fulfill our objectives. Because of the adjustment made in our methodological approach, we no longer have a need for animals and associated animal care costs. Only the anticipated costs of tissue culture are necessary. The Other expense category has also been reevaluated to help compensate for our staffing needs.
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**SUBTOTALS**

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|                        |                     |    |                |        |                    |        |
| **EQUIPMENT (Itemize)**|                     |    |                |        |                    |        |
| Laminar Flow Hood      | $7500               |    |                |        |                    |        |
| CO₂ Incubator          | 4500                |    |                |        |                    |        |
| Nikon Inverted Microscope | 11000           |    |                |        |                    |        |

**SUPPLIES (Itemize by category)**

- Tissue culture media, disposables, chemicals, CO₂, etc. $4910
- Microscopy photo supplies $2500
- EM chemicals $2500
- Biochemicals $5500
- Disposables, general chemicals, glassware $1500

**SUPPLIES TOTALS**: $16910

**DOMESTIC**

|                        |                     |    |                |        |                    |        |
| **CONSORTIUM/CONTRACTUAL COSTS** |                     |    |                |        |                    |        |

**OTHER EXPENSES (Itemize by category)**

- Blood donations $600
- Typing, page charges, reprints illustrations $1000
- Books, library services $400
- EM facility recharges $2000

**INDIRECT COSTS**: (From original proposal)

**TOTAL COSTS**: $152446