Medical College of Virginia
Virginia Commonwealth University

February 4, 1985

Mr. Perry A. Chapdelaine, Sr.
Executive Director/ Secretary
The Rheumatoid Disease Foundation
Rt. 4, Box 137
Franklin, TN 37064

Dear Mr. Chapdelaine:

I was pleased to meet with you and the other representatives of the Rheumatoid Disease Foundation during your December 14th visit to the Medical College of Virginia. I came away from the meeting with the appreciation that the Rheumatoid Disease Foundation is motivated to pursue new alternatives in rheumatoid disease.

Discussion during the meeting centered on the types of studies required to afford evidence to support or refute the Wyburn-Mason hypothesis. At the end, my understanding was that the Rheumatoid Disease Foundation would be interested in promoting this line of research. Therefore I am submitting the enclosed proposal, incorporating and expanding upon the substance of our discussion, in application for support from the Rheumatoid Disease Foundation to undertake studies relevant to the Wyburn-Mason hypothesis.

I look forward to the expeditious review of this proposal by the Rheumatoid Disease Foundation and to a fruitful collaborative venture.

Sincerely,

Brian M. Susskind

Brian M. Susskind, Ph.D.
Assistant Professor
Surgery and Microbiology
Medical College of Virginia
Box 629 - MCV Station
Richmond, VA 23298

BMS/ecb

APPLICATION FOR A GRANT FROM THE RHEUMATOID DISEASE FOUNDATION

Application is hereby made for a grant in the amount of $106,383 for the period June 1, 1985 through May 31, 1987 for the purpose of conducting a research project on the following subject:

Title of Project: Studies to Test the Wyburn-Mason Hypothesis on the Etiology of Rheumatoid Disease

Name of Principal Investigator: Brian M. Susskind, Ph.D.

Title of Principal Investigator: Assistant Professor of Surgery and Microbiology

Institution: Medical College of Virginia, Virginia Commonwealth University

Address: P.O. Box 629, MCV Station, Richmond, Virginia 23298-0001

Telephone: (804) 786-9663

PROPOSAL

Sponsor: Rheumatoid Disease Foundation

Title of Project: Studies to Test the Wyburn-Mason Hypothesis on the Etiology of Rheumatoid Disease

Project Period: June 1, 1985 - May 31, 1987

Total Costs: $106,383

APPLICANT ORGANIZATION:

Virginia Commonwealth University
Box 568, MCV Station
Richmond, Virginia 23298

IRS #: 54600-1758

BUSINESS OFFICE: (to whom checks are sent)

Margie R. Booker, Director,
Grants and Contracts Accounting
Virginia Commonwealth University
Box 552, MCV Station
Richmond, Virginia 23298

OFFICIAL APPROVING FOR VCU:

Herbert B. Chermside, Director,
Sponsored Programs Administration
Virginia Commonwealth University
Box 568, MCV Station
Richmond, Virginia 23298

PRINCIPAL INVESTIGATOR:

Brian M. Susskind, Ph.D.
Assistant Professor of
Surgery and Microbiology
Box 629, MCV Station
Richmond, Virginia 23298

Herbert B. Chermside
Authorized Official

Brian M. Susskind
Principal Investigator

A. INTRODUCTION

In recent years, a great deal of interest has been focused on a group of small, free-living amebae belonging to the genera Acathamoeba and Naegleria of the order Amoebidae. These organisms, which normally occur in fresh water and soil, are capable of facultative parasitism in man. The parasitic nature of these amebae was first recognized when trophozoites of Acanthamoeba were found contaminating monkey-kidney cell cultures (Jahnes et al., 1957; Chi et al., 1959). The organisms were suspected as being contaminants in the tissue cultures derived from airborne cysts. Acanthamoeba and Naegleria have been isolated, however, from throat and nasal swabs of patients with fever and respiratory symptoms, and from seemingly healthy individuals as well (Wang and Feldman, 1961, 1967; Shumaker et al., 1971). At least one species, Naegleria fowleri, has been shown to be highly pathogenic in man, causing primary amebic meningo-encephalitis.

It was in view of these observations--that free-living amebae are not entirely non-pathogenic, but are able to infect man--that Dr. R. Wyburn-Mason originally speculated about the possible role of free-living amebae in human disease (Wyburn-Mason, 1964). Indeed, Wyburn-Mason claimed to have isolated amebae from tissues in a number of disease states, including rheumatoid arthritis and cancer. Furthermore, Wyburn-Mason claimed success in treating these diseases with amebicidal drugs, especially rheumatoid disease (RD).

The concept of free-living amebae as a causative factor in arthritis, cancer, and other diseases is revolutionary, and the role of soil amebae in human diseases outside of primary amebic meningoencephalitis is not recognized by the medical establishment. It is unfortunate that the Wyburn-Mason hypothesis has not been subjected to the rigorous scientific scrutiny by which a hypothesis must be tested in order to become a tenet of modern medicine. Most of the "factual" support for th Wyburn-Mason hypothesis either has been published in books and journals that are not refereed, cited as "personal communications," or has been anecdotal. In this regard it is commendable that a double-blind clinical trial, supported by the Rheumatoid Disease Foundation, is being conducted at the Bowman-Gray School of Medicine to test the efficacy of amebicidal drugs in rheumatoid arthritis. Assuming the reputed efficacy of the amebicidal drugs in RD is borne out by these studies, this would still constitute only circumstantial evidence for amebal origin of RD. The mechanism of action for these drugs could involve biological effects other than their amebicidal activity. Additional research would be required to establish limax amebae as the etiologic agent in RD pathogenesis.

The criteria which are accepted by the scientific community as proof for the identification of a pathogen are contained in Koch's postulates:

1. The organism is regularly found in lesions of the disease.

2. It can be isolated in pure culture on artificial media.

3. Inoculation of this culture produces a similar disease in experimental animals.

4. The organism can be recovered from lesions of these animals.

This sort of systematic approach must be undertaken if sound, well-grounded evidence is to be produced to substantiate the assertion of Wyburn-Mason that RD is caused by pathogenic free-living amebae. In the two years' time we have allotted for this investigation it would be unrealistic to expect that all of Koch's postulates could be satisfied. The studies outlined in this proposal are designed to apply the first two, which are the more crucial, in an attempt to provide factual support for the Wyburn-Mason hypothesis.

B. SPECIFIC AIMS

Specific Aim #1: To isolate and culture the limax ameba reputed by Wyburn-Mason to be the causative agent of RD.

Specific Aim #2: To prepare antisera and monoclonal antibodies against limax ameba isolates.

Specific Aim #3: To demonstrate facultative parasitic limax amebae in situ.

C. EXPERIMENTAL PLAN

1. Specific Aim #1: To isolate and culture the limax ameba reputed by Wyburn-Mason to be the causative agent of RD

a. Culture Techniques

The capacity to grow the reputed pathogen in vitro will satisfy the first of Koch's postulates, facilitate the screening of therapeutic drugs and the development of vaccines, and allow study of their mechanisms of pathogenesis, their taxonomic relationship to other pathogenic free-living amebae, and their biology.

We will attempt to isolate limax amebae from human tissues of RD and cancer patients by the protocol which Wyburn-Mason described (1978). Limax amebae were reported to be abundant in many types of malignant tumors, and morphologically indistinguishable from those found in RD (Wyburn-Mason, 1978). The source of human material will be tissue taken at biopsy or operation which is sent to pathology for diagnostic tests. Dr. William J. Frable, Professor of Pathology and head of the surgical pathology laboratory, has agreed to turn over the remainder of specimens to us after taking samples for diagnosis. Surgical pathology will thus serve as the "clearing house" and facilitate the procurement of human materials. To expedite further the timely receipt of specimens, physicians from the various clinical services involved have agreed to notify us when material will be available. Dr. Lawrence Schwartz (rheumatologist, and a consultant for this project) will inform us of the availability of synovial fluid aspirates; Dr. John Cardea (Chairman of Orthopedic Surgery) will inform us of the availability of tissue from rheumatoid and osteoarthritis patients undergoing joint replacement; and Drs. Walter Lawrence (Chairman of Surgical Oncology) and William Regelson (medical oncology, consultant) will inform us of the availability of tumor tissue. It is estimated that synovial fluid aspirates will be available at the rate of 50 per month; synovial tissues from operation will be available at the rate of 30 per month, with between 20% and 50% of these from RD patients; tumor tissues will be available on almost a daily basis. We will be

able to process and effectively deal with fifteen (15) samples of synovium per month, fifteen (15) synovial aspirates, and five (5) tumor samples [preferentially lymphomas (Wyburn-Mason, 1978)].

For the isolation procedure, the apparatus used by Wyburn-Mason to induce migration of the amebae out of the tissue, based on their thermotropic properties, will be reproduced. The tissue will be cooled to 0°C for two hours, after which time the solution in the lower half of the apparatus [Page's "ameba saline" (Page, 1967)], containing any cells which have migrated through the membrane filter, is collected into a sterile test tube. Centrifugation is reported to kill the organism (Wyburn-Mason, 1978). Therefore, we will let the tube sit on ice for 1-2 hours, allowing any cells in it to settle by gravity. (Other more rapid and equally gentle techniques for recovery of cells in the supernatant will be tested, such as centrifugation through cushions of Percoll forming a buoyant density gradient). All of the fluid except for 1 ml is aspirated off; the remainder is used to inoculate culture media.

Reported methods for the cultivation of pathogenic free-living amebae of various strains of Naegleria and Acanthamoeba should be helpful in successfully propagating isolated amebae. Pathogenic free-living amebae have been successfully grown on agar medium in association with bacteria on which the amebae feed (Singh, 1950; Chang, 1960). Wyburn-Mason used Noble agar plates carpeted with a live strain of Escherichia coli in his isolation protocol, but it is not clear whether growth of the amebae occurred in this medium (1964). The appearance of bacteriophage-like plaques in the lawn of bacteria will be a useful, easily visible sign of amebal growth. Direct microscopic examinations will be made on a daily basis to look for moving trophozoites and cysts. It may be necessary to try a variety of bacteria species as food, as the growth characteristics of amebae are affected by the bacterial associate (Chang, 1960). General experience shows that strains of Klebsiella/Aerobacter are preferable to E. coli (S.G. Bradley, Personal Communication, 1984).

It is more difficult to analyze and understand the biology of an organism when in vitro growth of that organism requires xenic cultivation. The presence of an associate "food" organism also complicates the production of antibodies directed specifically against the organism of interest. Several axenic culture media have been described for the pathogenic free-living amebae (Warhurst and Armstrong, 1968; Neff, 1957; Visvesvara and Balamuth, 1975; Nerad et al., 1983). Since it is usually easier to isolate pathogenic amebae (both free-living and obligate parasites such as Entamoeba histolytica) in xenic cultures, initial isolation procedures will employ culture media with bacterial associates. To establish axenic cultures, these culture media will be allowed to become dehydrated, inducing the amebae to encyst, and the cysts rendered free of contaminating bacteria by washing in antiseptic chemicals. Alternatives include weaning of the amebae successively from live bacteria to heat-killed bacteria to axenic cultures, and the use of an antibiotic sensitive bacterium in xenic cultures, with the addition of antibiotic upon transfer to axenic media to kill the bacterium.

Cultures will be placed in a Queue incubator equipped with a RG3 continuous flow gas mixing system. With this incubator a defined atmosphere can be established by proportional mixing of up to three gasses (e.g., nitrogen, oxygen, and carbon dioxide), allowing culture conditions to be optimized. Initial attempts will be made using air or air with 5% carbon dioxide. The incubators will be set at

body temperature (37°C) since non-pathogenic strains of Naegleria and Acanthamoeba are inhibited from growing at this temperature. All limax ameba isolates will be cryopreserved and stored in liquid nitrogen. We have established protocols for the successful cryopreservation of E. histolytica and numerous mammalian cell lines.

Safeguards must be taken to limit and control for the contamination of cultures by amebae or amebal cysts from external sources, e.g., infected tissue culture reagents, airborne cysts in the laboratory, the human nasopharynx. Safeguards to be employed include: 1) sterilization by autoclave of all apparatus; 2) filtration (0.2 u pore size) of all tissue culture media; 3) the use of sterile, disposable tissue culture plastics (commercially available); 4) the use of a biohazard containment hood for all isolation procedures and tissue culture work; 5) strict avoidance of mouth pipetting by use of air pump-driven pipetting aides equipped with filters; 6) running parallel uninoculated cultures as controls; 7) conducting sterility tests on all positive cultures since airborne contamination from the external environment would be expected to result in the uncontrolled introduction of other organisms besides encysted amebae. If no growth of bacteria (apart from the particular associate species in xenic cultures) or yeast is observed in the test media, external contamination can be ruled out.

Furthermore, that the origin of amebal isolates is the human tissues must be demonstrated by identification of the amebae in situ. Samples of all tissues used in attempts to isolate amebae will be frozen and stored in liquid nitrogen so that after a successful isolation, sections can be cut from the block of tissue for immunohistochemical staining (Specific Aim #3).

b. Taxonomic Identification

Identification of limax amebae isolated will be based on the morphology of trophozoites and cysts obtained from the initial isolation medium and from in vitro cultures, and based on their antigenic content, determined using anti-amebal antibodies raised against strains of Naegleria and Acanthamoeba from pure cultures (Visvesvara and Balamuth, 1975). Thus, their relationship to the other pathogenic free-living amebae in the family Amoebidae will be established, and it will be of considerable interest to determine whether limax amebae obtained from RD tissues and malignant tissues are of the same species. The organism isolated by Wyburn-Mason was tentatively classified as belonging to the genus Naegleria based on morphologic features of the trophozoites and cysts, the formation of cysts in vitro but not in vivo, and its development into a bi-flagellate form in distilled water (Wyburn-Mason, 1978).

2. Specific Aim #2: To prepare antisera and monoclonal antibodies against limax ameba isolates

Assuming that we are successful in isolating and culturing limax amebae from diseased human tissues as described above, we will next prepare antisera and monoclonal antibodies against the organism for use in further studies (e.g., Specific Aim #3). Amebae grown in axenic cultures are the preferred source of antigen; otherwise, thorough washing and differential sedimentation through Percoll will be used to remove most of the contaminating bacterial associate from the amebae. Antisera will be raised in white albino rabbits using antigen emulsified in Freund's complete adjuvant. Antibody titers will be monitored using the agglutination test (Reilley et al., 1982).

Antisera will also be raised against known strains of Naegleria and Acanthamoeba for studies to compare the antigenic profile of these strains with the Wyburn-Mason-type limax ameba in order to evaluate their taxonomic relationship.

Monoclonal antibodies will also be produced against the limax amebae. Relative to monoclonal antibodies, the specific antibodies obtained by conventional immunization are faster, simpler, and cheaper to prepare. The advantages of monoclonal antibodies over the polyclonal antibodies obtained by conventional immunization, however, are in their defined specificity, homogeneity, and availability in practically unlimited quantities. Anti-limax ameba monoclonal antibodies would be initially used in Specific Aim #3. A further application for these monospecific reagents would be the purification of the antigens which they recognized. Obviously these would be among the immunogenic amebal antigens, and as such would be useful in the eventual development of an anti-limax ameba vaccine.

The methodology involved in the derivation of monoclonal antibodies is well established, does not require overly sophisticated procedures or expensive equipment, and has been employed by this investigator. Furthermore, the Medical College of Virginia established a Hybridoma Laboratory in January, 1983, as a shared research resource to provide reagents, cell lines, and technological expertise to facilitate the production of monoclonal antibodies.

3. Specific Aim #3: To demonstrate facultative parasitic limax amebae in situ

Crucial for the validation of the Wyburn-Mason hypothesis would be the demonstration of the causative organism in situ. It is an important enigma that the organisms described by Dr. Wyburn-Mason were not observed in tissue specimens from which the organisms had been isolated. Positive identification of amebae in tissue sections can be difficult for a number of reasons: 1) amebae (e.g., E. histolytica) tend to be unevenly distributed, requiring that several blocks of tissue be examined; 2) morphologically, amebae may resemble leukocytes; and 3) amebae are unrecognizable in tissue unless the plane of the cut includes the nucleus.

Immunofluorescent staining is a powerful tool used in research and in diagnostic work for detecting organisms that are not normally observable in tissue stained by ordinary methods. We will attempt immunofluorescent staining to demonstrate amebae in specimens of rheumatoid tissue using the indirect or "sandwich" technique. Tissue sections are first reacted with immune serum containing a high titre of anti-limax ameba antibody, then with a second antibody, conjugated to a fluorescent dye (e.g., fluorescein-isothiocyanate (FITC), directed against immunoglobulin from the animal species used as the source of immune serum. If amebae are present in the tissue, they will be bound by the anti-ameba antibody, which will in turn be bound by the FITC-conjugated anti-antibody reagent. The specimens are examined with a fluorescence microscope.

As a source of primary immune serum, the first choice is antiserum raised in rabbits or a monoclonal antibody produced in mice against amebae isolated from rheumatoid patients (Specific Aim #2). If these cannot readily be produced because the amebae prove difficult to isolate or culture, an alternative source of immune serum would be patients with a high titer of anti-Naegleria agglutinating antibody (Reilley et al., 1982, 1983). Alternatively, antisera raised in rabbits

against defined strains of Naegleria and Acanthamoeba would be employed. Although a less desirable source of antiserum than either of the former two, the demonstration of a positive staining reaction with these antibodies would be good evidence for the presence of pathogenic amebae. The presence of cross-reacting common antigens is observed among the species within each genera (Visvesvara and Balamuth, 1975).

It is appreciated by this investigator that a number of parameters will have to be worked out in order to establish the technique for these organisms and with the reagents available. We will establish the technique (e.g., dilutions of reagents, time, temperature, etc., to be used for optimal staining) with tissue sections from the brains of mice experimentally infected with N. fowleri, and anti-Naegleria antisera. The specimens of tissue to be examined will be prepared by frozen section since processing of tissues by formalin fixation may destroy relevant antigens. For controls, sections cut from the same tissue blocks are reacted with non-immune serum as the primary antibody.

This method will provide a means of rapidly screening a large number of tissue specimens for the presence of amebae without the need for the amebae to have been fortuitously cut in a plane so that they were left morphologically identifiable. Of course, confirmation will require that amebae recognizable with hemotoxylin be demonstrated. Double-staining with hemotoxylin and fluorescent antibody can be performed and photomicrographs taken with both fluorescent microscopy and light microscopy. Objects identified with hemotoxylin stain as amebae should be coincidentally fluorescent, as demonstrable by superimposition of the micrographs. Ultimately, it would be necessary to isolate amebae from the same block of tissue in which they are observed by immunofluorescence.

D. EXPERIENCE OF THE PRINCIPAL INVESTIGATOR RELEVANT TO THIS PROJECT

As the principal investigator, I will have the overall responsibility of directing the progress of this investigation. Both my M.S. and Ph.D. are in the field of Tropical Medicine and Medical Parasitology. My research leading to the M.S. and Ph.D. degrees involved biochemical studies on the human amebal parasite, E. histolytica. Three years of postdoctoral study were spent at the Sloan Kettering Institute for Cancer Research in the Department of Immunobiology. At present in my laboratory at the Medical College of Virginia, we are investigating the immunobiology of certain protozoan parasitic diseases. One project is a study of the mucosal immune response to E. histolytica which is being conducted in collaboration with Dr. Lawrence Schwartz, a rheumatologist and consultant for the proposed investigation. Our findings on the immunologic and inflammatory processes mediated by leukocytes reacting to invasion of the large intestine by E. histolytica might well have relevance to the pathogenesis of rheumatoid disease. Thus, my background training and experience are especially appropriate for the proposed investigation. Furthermore, the laboratory infrastructure in terms of tools, trained technical personnel, and collaborators is in place and will allow expeditious development of this project.

E. RESOURCES AND ENVIRONMENT

Dr. Brian Susskind's laboratory on the 9th floor of Sanger Hall (MCV/VCU) contains about 800 square feet of space and includes a 160 square foot tissue culture room. The following are available in the P.I.'s laboratory: laminar flow hood, CCI biohazard hood (6 ft.), refrigerated floor and table-top centrifuges, microscopes, 2 Queue RG3 incubators, pH meter, water baths, refrigerator, freezer, and complete bench-top facilities. Equipment not physically located in the laboratory but available within the department on the same floor as his laboratory include complete histologic facilities for the preparation of fresh cryostat and fixed sections, fluorescence microscope, controlled rate liquid nitrogen cryostat freezer, liquid nitrogen freezers, cold rooms, and an Ortho Spectrum III cytofluorograph. All support services--animal maintenance, glassware washing, media preparation, machine and repair shop, testing laboratories, etc., are available within Sanger Hall. Office space of 125 square feet with excellent secretarial and word processing capabilities is available for these studies within the Department of Surgery on the 8th floor of Sanger Hall. As the P.I. is a member of the faculty of the Department of Surgery, Division of Surgical Oncology, excellent departmental and interdepartmental cooperation can be anticipated in obtaining human tissue at biopsy, operation, or autopsy.

Consultants who have agreed to assist with this investigation are: Dr. William Regelson, M.D., Professor of Internal Medicine and Oncology; Dr. Gaylen Bradley, Professor of Microbiology and Immunology, and Dean of the School of Basic Science; Dr. Lawrence Schwartz, Associate Professor of Immunology and Connective Tissue Diseases, and Chief of Allergy and Immunology, and Dr. William J. Frable, Professor of Pathology. The former three individuals met with representatives from the Rheumatoid Disease Foundation during their recent visit to the Medical College of Virginia.

F. PROPOSED BUDGET

	1st Year	2nd Year	
Personnel			
Brian Susskind, Principal Investigator (10% of time and effort)	$ 3,800	$ 4,028	
Elissa Dixon, Lab. Tec. B (100% of time and effort)	20,646	21,885	
(increased by 6% for second year to offset merit and cost-of-living raises)			
Retirement and other benefits (28.40)	6,943	7,481	(28.87%)
Permanent Equipment			
Inverted microscope	8,000		
Consumable Supplies			
Tissue culture supplies	4,000	4,400	
Tissue culture medium	4,000	4,400	
FITC-conjugated antibodies	2,000	2,200	
Animal maintenance	3,000	3,300	
Miscellaneous glassware	1,000	1,100	
Miscellaneous chemicals	1,000	1,100	
Publication costs	1,000	1,100	
(increased by 10% for second year)			
Total	$55,389	$50,994	

REFERENCES

1. Chang, S.L.: Can. J. Microbiol. 6:397-405, 1960.

2. Chi, L., Vogel, J.E., and Shilokov, A.: Science 130:1763, 1959.

3. Jahnes, W.G., Fullmer, H.M., Li, C.P.: Proc. Soc. Exp. Biol. Med. 96:484-488, 1957.

4. Neff, R.J.: J. Protozool. 4:176-182, 1957.

5. Nerad, T.A., Visvesvara, G., Daggett, P.-M.: J. Protozool. 30:383-387, 1983.

6. Page, F.: J. Protozool. 14:499, 1967.

7. Reilly, M.F., Bradley, M.K., and Bradley, S.G.: Proc. Soc. Exp. Biol. Med. 170:209-212, 1982.

8. Reilly, M.F., Marciano-Cabral, F., Bradley, D.W., and Bradley, S.G.: J. Clin. Microbiol. 17:576-581, 1983.

9. Shumaker, J.B., Healy, G.R., English, D., Schultz, M., and Page, F.C.: Lancet II:602-603, 1971.

10. Singh, B.N.: Nature 165:65, 1950.

11. Visvesvara, G.S., and Balamuth, W.: J. Protozool. 22:245-256, 1975.

12. Wang, S.S. and Feldman, H.A.: Antimicrobiol. Ag. Chemother. 8:50-53, 1961.

13. Wang, S.S., and Feldman, H.A.: N. Engl. J. Med. 227:1174-1179, 1967.

14. Warhurst, D.C., and Armstrong, J.A.: J. Gen. Microbiol. 50:207-215, 1968.

15. Wyburn-Mason, R.: Medical Hypothesis 5:1237-1249, 1979.

16. Wyburn-Mason, R.: The Causation of Rheumatoid Disease and Many Human Cancers. A New Concept in Medicine. IJI Publ. LTD, Tokyo, Japan, 1978.

17. Wyburn-Mason, R.: A New Protozoan. Its Relation to Malignant and Other Diseases. Kimpton, London, 1964.