U.S. AGENCY FOR
INTERNATIONAL
DEVELOPMENT

ENTERED

OCT 27 1993

Section

Dr. Edward L. Risby, Executive Associate Dean
School of Medicine
Meharry Medical College
1005 D.B. Todd Boulevard
Nashville, TN 37208

Subject: Grant No. PCE-5053-G-00-3048-00

Dear Dr. Risby:

Pursuant to the authority contained in the Foreign Assistance Act of 1961 and the Federal Grant and Cooperative Agreement Act of 1982, as amended, the Agency for International Development (hereinafter referred to as "A.I.D.") hereby grants to Meharry Medical College (hereinfter referred to as "MMC" or "the Grantee") the sum of $98,835 to provide financial support for the program described in Attachment 2 of this Grant entitled "Program Description."

This Grant is effective and obligation is made as of the date of this letter and shall apply to expenditures made by the Grantee in furtherance of program objectives during the period beginning with the effective date and ending September 30, 1995.

This Grant is made to the Grantee on the condition that the funds will be administered in accordance with the terms and conditions as set forth in Attachment 1 (the Schedule); Attachment 2 (the Program Description); and Attachment 3 (the Standard Provisions); all of which have been agreed to by your organization.

Please sign the original and all enclosed copies of this letter to acknowledge your receipt of this grant and return the original and all but one copy to the undersigned.

If you have any questions, please contact Ms. Karin Kolstrom of my staff at (703) 875-1189.

Sincerely,

Gary V. Kinney

Gary V. Kinney
Grant Officer
Chief, PCE Branch
Division B
Office of Procurement

320 TWENTY-FIRST STREET, N.W., WASHINGTON, D.C. 20523

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Attachments:

1. Schedule
2. Program Description
3. ~~Standard Provisions~~

ACKNOWLEDGED:

Meharry Medical College

BY: Edward L. Risby

TYPED NAME: Edward L. Risby, Ph.D.

TITLE: Executive Associate Dean, School of Medicine

DATE: 9-28-93

97381

FISCAL DATA

A. GENERAL

A.1. Total Estimated A.I.D. Amount: ~~$100,000~~ $98,835
A.2. Total Obligated A.I.D. Amount: $98,835
A.3. Cost-Sharing Amount (Non-Federal): $0
A.4. Other Contributions (Federal): $0
A.5. Project No.: 936-5053
A.6. A.I.D. Project Office: R&D/UC
A.7. Funding Source: A.I.D./W
A.8. Tax I.D. No.: 64-0488046
A.9. CEC No.: 04-143-8185
A.10. LOC No.: 72-00-1540

B. SPECIFIC

B.1.(a) PIO/T No.: 936-5053-3692942
B.1.(b) Appropriation: 72-1131021.1
B.1.(c) Allotment: 341-36-099-00-20-31
B.1.(d) BPC: DDVA-93-16900-KG11
B.1.(e) Amount: $98,835

ATTACHMENT 1

SCHEDULE

1A. PURPOSE OF GRANT

The purpose of this Grant is to provide financial support for the program described in Attachment 2 of this Grant entitled "Program Description."

1B. PERIOD OF GRANT

The effective date of this Grant is the date of the Cover Letter and the estimated completion date is September 30, 1995. Funds obligated hereunder (see Section 1C.2. below) shall be used to reimburse the Grantee for allowable program expenditures incurred by the Grantee in pursuit of program objectives during such period. Funds obligated hereunder are anticipated to be sufficient for completion by the Grantee of the program described in Attachment 2 of this Grant by the estimated completion date.

1C. AMOUNT OF GRANT AND PAYMENT

1C.1. The total estimated amount of this Grant for its full period, as set forth in Section 1B. above, is $98,835.

1C.2. A.I.D. hereby obligates the amount of $98,835 for the purposes of this Grant during the indicated period set forth in Section 1B. above, thereby fulfilling A.I.D.'s funding requirements. A.I.D. shall not be liable for reimbursing the Grantee for any costs in excess of the obligated amount, except as specified in paragraph (f) of the Standard Provision of this Grant entitled "Revision of Grant Budget."

1C.3. Payment shall be made to the Grantee in accordance with procedures set forth in the Standard Provision of this Grant entitled "Payment - Letter of Credit," as shown in Attachment 3.

1D. GRANT BUDGET

1D.1. The following is the Budget for the total estimated amount of this Grant (see Section 1C.1. above) for its full period (see Section 1B. above). The Grantee may not exceed the total estimated amount or the obligated amount of this Grant, whichever is less (see Sections 1C.1. and 1C.2., respectively, above). Except as specified in the Standard Provision of this Grant entitled "Revision of Grant Budget," as shown in Attachment 3, the Grantee may adjust line item amounts as may be reasonably necessary for the attainment of program objectives.

BEST AVAILABLE DOCUMENT

1D.2. Budget

Cost Element	A.I.D.	Cost-Sharing Non-Federal	Total
Salaries	$37,944	$0	$37,944
Fringe	$7,589	$0	$7,589
Travel	$1,000	$0	$1,000
Non-Exp. Equip.	$0	$0	$0
ODC	$23,513	$0	$23,513
Overhead	$28,789	$0	$28,789
Total:	$98,835	$0	$98,835

1D.3. Inclusion of any cost in the budget of this Grant does not obviate the requirement for prior approval by the Grant Officer of cost items designated as requiring prior approval by the applicable cost principles (see the Standard Provision of this Grant set forth in Attachment 3 entitled "Allowable Costs") and other terms and conditions of this Grant, unless specifically stated in Section 1I. below.

1E. REPORTING

1E.1. Financial Reporting

1E.1.(a) Financial reporting requirements shall be in accordance with the Standard Provision of this Grant entitled "Payment - Letter of Credit," as shown in Attachment 3. If a Standard Form 269 is required by the aforesaid Standard Provision, the "Long Form" of said form shall be used.

1E.1.(b) All financial reports shall be submitted to A.I.D., Office of Financial Management, FA/FM/CMPD/DCB, Room 700 SA-2, Washington, D.C. 20523-0209. In addition, three copies of all financial reports shall be submitted to the A.I.D. Project Office specified in the Cover Letter of this Grant, concurrently with submission of the Quarterly Technical Reports (See Section 1E.2. below).

1E.1.(c) The frequency of financial reporting and the due dates of reports shall be as specified in the Standard Provision of this Grant referred to in Section 1E.1.(a) above.

1E.2. Program Reporting

1E.2.(a) Annual Workplan

1E.2.(a)(1) The Grantee shall submit an annual workplan for this Grant which shall contain the following:

1E.2.(a)(1)(A) An action-oriented workplan describing planned activities for the next year, delineated by calendar quarter, and linked to the project goals and objectives, which describes the individuals to be involved, the activities to be conducted, and where and when they will be conducted. Planned activities shall be grouped by subject category, and then related to project objectives;

1E.2.(a)(1)(B) A projected budget, utilizing the same budget line items as are set forth in the budget of this Grant, for each calendar quarter, corresponding to the workplan; and

1E.2.(a)(1)(C) Publications, reports, workshops, seminars, and other information dissemination activities planned, by calendar quarter.

1E.2.(a)(2) The Grantee may develop the annual workplan in consultation with the A.I.D. Project Officer for this Grant.

1E.2.(a)(3) Five (5) copies of the annual workplan shall be submitted to the designated A.I.D. Project Officer for this Grant and one copy submitted to the Grant Officer. The annual workplan shall be submitted by the Grantee not later than sixty (60) days from the effective date of this Grant (see Section 1B. above).

1E.2.(b) Quarterly Reports

The Grantee shall submit five (5) copies of brief quarterly program performance reports, which coincide with the financial reporting periods described in Section 1E.1. above, to the A.I.D. Project Office specified in the Cover Letter of this Grant. In addition, two copies shall be submitted to A.I.D., POL/CDIE/DI, Washington, DC 20523-1802. These reports shall be submitted within 30 days following the end of the reporting period, and shall briefly present the following information:

1E.2.(b)(1) A comparison of actual accomplishments with the goals established for the period, the findings of the investigator, or both. If the output of programs can be readily quantified, such quantitative data should be related to cost data for computation of unit costs.

1E.2.(b)(2) Reasons why established goals were not met, if applicable.

1E.2.(b)(3) Other pertinent information including the status of finances and expenditures and, when appropriate, analysis and explanation of cost overruns or high unit costs.

BEST AVAILABLE DOCUMENT

1E.2.(c) Special Reports

1E.2.(c)(1) Within 30 days following the completion of each international trip, the Grantee shall submit 3 copies of a trip report summarizing the accomplishments of the trip to the A.I.D. Project Officer specified in the cover letter of this Grant. If several individuals are travelling together to one site, a single report representing the group will suffice. The report shall include the purpose of the trip, technical observations, suggestions and recommendations, overall impressions of the site situation (if appropriate), and a list of persons visited with their title and organization affiliation.

1E.2.(c)(2) Between the required program performance reporting dates, events may occur that have significant impact upon the program. In such instances, the Grantee shall inform the A.I.D. Project Officer as soon as the following types of conditions become known:

1E.2.(c)(2)(A) Problems, delays, or adverse conditions that will materially affect the ability to attain program objectives, prevent the meeting of time schedules and goals, or preclude the attainment of work units by established time periods. This disclosure shall be accompanied by a statement of the action taken, or contemplated, and any A.I.D. assistance needed to resolve the situation.

1E.2.(c)(2)(B) Favorable developments or events that enable time schedules to be met sooner than anticipated or more work units to be produced than originally projected.

1E.2.(c)(3) If any performance review conducted by the Grantee discloses the need for change in the budget estimates in accordance with the criteria established in the Standard Provision of this Grant entitled "Revision of Grant Budget," the Grantee shall submit a request for budget revision to the Grant Officer and the A.I.D. Project Officer specified in the Cover Letter of this Grant.

1E.2.(d) Environmental Impact

If it appears that the outputs of this project will result in an adverse environmental impact, the Grantee shall notify the A.I.D. Project Officer prior to implementation, in order to allow for orderly preparation of an environmental impact statement. The Grantee shall assure that appropriate U.S. Government and/or host country procedures are followed.

1E.2.(e) Care of Laboratory Animals

If the Standard Provision entitled "Care of Laboratory Animals" applies to this Grant, the Grantee shall include the certificate required by paragraph (c) of said Standard Provision in all of its reports which pertain to the use of laboratory animals.

1E.2.(f) Compliance With Federal Guidelines and Regulatory Procedures Pertaining to Recombinant DNA

The Grantee shall implement any research activities under this Grant which involve recombinant DNA in accordance with: 1) the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules; 2) procedures issued by the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA), or other appropriate Federal agency; 3) A.I.D.'s environmental procedures; and 4) such other Federal guidelines and procedures as may apply during the course of research. The Grantee cannot commence testing in any foreign location until written approval for such testing is obtained from the A.I.D. Project Officer and the government of the country where testing is planned. Testing shall be conducted in accordance with all applicable regulations of that country. In addition, and prior to commencement of any such testing, the Grantee shall make a judgement and communicate same to the A.I.D. Project Officer as to whether the regulations, procedures, or facilities of the country in question are adequate to ensure testing in an environmentally sound manner. In the event such judgement is that they are not, the Grantee and the A.I.D. Project Officer will consult and agree on the conditions to be applied to the testing which will have such environmental effect. Reports submitted to A.I.D. under this Grant will address regulatory issues as noted above related to the activity.

1E.2.(g) Final Report

Within 90 days following the estimated completion date of this Grant (see Section 1B. above), the Grantee shall submit five (5) copies of a final report to the A.I.D. Project Office specified in the cover letter of this Grant. In addition, two copies shall be submitted to A.I.D., POL/CDIE/DI, Washington, DC 20523-1802. It will cover the entire period of the Grant and include all information shown in Sections 1E.2.(a) and 1E.2.(c) above.

1F. SPECIAL PROVISIONS

1F.1. OPTIONAL STANDARD PROVISIONS

The following Optional Standard Provisions for U.S., Nongovernmental Grantees, as listed in Attachment 3 of this Grant, are hereby deleted as follows:

Provision	Page
Payment - Periodic Advance	13
Payment - Cost Reimbursement	15
Local Cost Financing	33
Patent Rights	35
Negotiated Indirect Cost Rates - Provisional	46
Participant Training	48
Voluntary Population Planning	49
Protection of the Individual as a Research Subject	56
Title To and Care of Property(U.S. Government Title)	63
Title To and Care of Property(Cooperating Country Title)	67
Cost Sharing	71

All other Optional Standard Provisions are hereby incorporated into this Grant and have been checked off on page 4C-9 of Attachment 3.

1F.2. Limitations on Reimbursement of Costs of Compensatior for Personal Services and Professional Service Costs

1F.2.(a) Employee Salaries

Except as the Grant Officer may otherwise agree in writing, A.I.D. shall not be liable for reimbursing the Grantee for any costs allocable to the salary portion of direct compensation paid by the Grantee to its employees for personal services whic exceed the highest salary level for a Foreign Service Officer, Class 1 (FS-1), as periodically amended.

1F.2.(b) Consultant Fees

Compensation for consultants retained by the Grantee hereunder shall not exceed, without specific approval of the rate by the Grant Officer: either the highest rate of annual compensation received by the consultant during any full year of the immediately preceding three years; or the maximum rate of a Foreign Service Officer, Class 1 (FS-1) (as periodically amended), whichever is less. A daily rate is derived by dividing the annual compensation by 2,087 and multiplying the result by 8.

1F.3. Defense Base Act (DBA) and Medical Evacuation Insurance

Pursuant to Section J.16. of OMB Circular A-21 (for educational institutions) or Section 18 of Attachment B of OMB Circular A-122 (for nonprofit organizations other than educational institutions), the Grantee is authorized to purchase DBA and/or medical evacuation insurance under this Grant. If DBA insuranc is purchased, it shall be purchased from the insurance company or agent with which A.I.D. has a contract to provide DBA insurance for A.I.D. contracts. The Grant Officer will provide the name, address, and telephone number of such insurance company or agent upon request.

1G. INDIRECT COST RATE

Pursuant to the Standard Provisions of this Grant entitled "Negotiated Indirect Cost Rates - Predetermined" and "Negotiated Indirect Cost Rates - Provisional (Nonprofits)," a predetermined indirect cost rate or rates shall be established for each of the Grantee's accounting periods which apply to this Grant. Pending establishment of predetermined indirect cost rates for the initial period (9/30/93 - 9/29/95), provisional payments on account of allowable indirect costs shall be made on the basis of the following negotiated provisional rate(s) applied to the base(s) which is (are) set forth below:

Type	Rate	Applicability	Base
On-Campus/Home Office	40.9%	Instruction	1/
"	41.1%	Research	1/
"	31.0%	Other Spons. Progs.	1/
Off-Campus/Off-Site	N/A		

1/ Base of Application: Modified total direct costs consisting of salaries and wages, fringe benefits, materials and supplies, services, travel and subawards up to $25,000 each.

1G.1. Rates for subsequent periods shall be established in accordance with the Standard Provision of this Grant entitled "Negotiated Indirect Cost Rates - Predetermined."

1H. PROPERTY REQUIREMENTS

1H.1. Title to Property

Title to property acquired hereunder shall vest in the Grantee subject to the requirements of the Standard Provision of this grant entitled "Title To and Use of Property (Grantee Title)" regarding use, accountability, and disposition of such property.

1H.2. Equipment Purchases

Equipment purchases under this Grant must be made in accordance with the Standard Provisions entitled "Procurement of Goods and Services" and "AID Eligibility Rules for Goods and Services" included in Attachment 3 of this Grant. Inclusion of costs in the budget of this Grant for the purchase of nonexpendable equipment does not obviate the requirements of Section J.13. of OMB Circular A-21 (for educational institutions) or Section 13 of Attachment B of OMB Circular A-122 (for nonprofit organizations other than educational institutions) for prior approval of such purchases by the Grant Officer, nor any other terms and conditions of this Grant, unless specifically stated in Section 1H.2.(a)(3) below.

1H.2.(a) Requirement for Prior Approval

Pursuant to Sections 1D.3. and 1G.3. above and the Standard Provisions of this Grant entitled "Allowable Costs" and "Revision of Grant Budget," and by extension, Section 13 of Attachment B of OMB Circular A-122, the Grantee must obtain A.I.D. Grant Officer approval for purchases of the following:

1H.2.(a)(1) General Purpose Equipment, which is defined as an article of nonexpendable tangible personal property which is usable for other than research, medical, scientific or technical activities, whether or not special modifications are needed to make them suitable for a particular purpose (e.g., office equipment and furnishings, air conditioning equipment, reproduction and printing equipment, motor vehicles, and automatic data processing equipment), having a useful life of more than two years and an acquisition cost of $500 or more per unit); and

1H.2.(a)(2) Special Purpose Equipment, which is defined as an article of nonexpendable tangible personal property, which is used only for research, medical, scientific, or technical activities (e.g., microscopes, x-ray machines, surgical instruments, and spectrometers), and which has a useful life of more than two years and an acquisition cost of $1,000 or more per unit).

1H.2.(a)(3) Approvals

In furtherance of the foregoing, the Grant Officer does hereby provide approval for the following purchases, which shall not be construed as authorization to exceed the total estimated amount or the obligated amount of this Grant, whichever is less (see Section 1C. above):

N/A

1H.2.(a)(4) Exception for Automation Equipment

Any approval for the purchase of automation equipment which may be provided in Section 1H.2.(a)(3) above or subsequently provided by the Grant Officer is not valid if the total cost of purchases of automation equipment (e.g., computers, word processors, etc.), software, or related services made hereunder will exceed $100,000. The Grantee must, under such circumstances, obtain the approval of the Grant Officer for the total planned system of any automation equipment, software, or related services.

II. RESOLUTION OF CONFLICTS

Conflicts between any of the Attachments of this Grant shall be resolved by applying the following descending order of precedence:

Attachment 1 - Schedule
Attachment 3 - Standard Provisions
Attachment 2 - Program Description

AGENCY FOR INTERNATIONAL DEVELOPMENT
RESEARCH GRANTS PROGRAM
FOR
HISTORICALLY BLACK COLLEGES AND UNIVERSITIES

Meharry Medical College
School of Medicine
Department of Microbiology
1005 D.B. Todd Blvd.
Nashville, TN 37208

The construction and screening of a *Plasmodium yoelii* cDNA expression library in an attenuated *Salmonella typhimurium* vaccine strain

(New Submission)

Principal Investigator:

James M. Burns, Jr.
Assistant Professor
Meharry Medical College
Department of Microbiology
1005 D.B. Todd Blvd.
Nashville, TN 37208

April 30, 1993

James M Burns Jr.
James M. Burns, Jr., Ph.D.
Department of Microbiology
Phone : (615)-327-5726
FAX : (615)-327-6072

Edward L. Risby
Edward L. Risby, Ph.D.
Executive Associate Dean
School of Medicine

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I. Other Support

The proposed research has not been submitted to other funding organizations nor is it closely related to research being done for other sponsors in whole or in part.

II. Need for the Proposed Research

The U.S. Agency for International Development (A.I.D.) has long recognized that the morbidity and mortality due to a number of parasitic diseases is a major impediment to the development of many countries in the tropical areas of the world. This is particularly true for malaria where the number of individuals today who are infected or at risk of infection with Plasmodium falciparum may never have been greater. This is largely due to the emergence and spread of insecticide-resistant mosquito vectors and drug-resistant plasmodia combined with the escalating expense of these control measures. This is clearly a major public health issue with significant economic and social consequences. One possible solution which has been given considerable attention has been the development of an effective multivalent malaria vaccine.

The challenge of the malaria vaccine effort has been 1) to identify protective antigens; 2) to produce and purify these antigens in an appropriate recombinant and/or synthetic form; and 3) to immunize with these antigens with the correct dose, route and adjuvant in order to induce protective immunity. A number of plasmodial proteins have been identified as targets of the host immune response during infection in man or in animal models. Often, this identification process has involved either antibody or T cell reactivities of individuals who have resolved their infections. Parasite proteins believed to be functionally important such as those involved in plasmodial invasion of hepatocytes or erythrocytes have also been pursued as vaccine targets. It is not apparent that these strategies have lead to the identification of the best vaccine components. In addition, difficulties have been encountered in producing and delivering these candidate antigens in an appropriate form to be effective in inducing protection against disease. Some level of success may be possible with the plasmodial antigens presently under consideration for vaccine use. To extend these efforts, however, it is clear that other strategies and additional malaria vaccine candidates need to be pursued.

We plan to take a slightly different approach to this problem. The broad objective of the present proposal is to lay the foundation for the development of a recombinant attenuated Salmonella vaccine for P. falciparum malaria. We are not taking the traditional approach of expressing a specific antigen gene in Salmonella in order to evaluate its immunizing potential. The specific goal of this proposal is to develop a system using the Plasmodium yoelii model to identify protective malaria antigens by constructing a blood-stage cDNA expression library in an attenuated Salmonella typhimurium

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strain and screening recombinants for their ability to protect mice against lethal P. yoelii infection. The appeal of this approach is that it is designed to identify protective plasmodial antigens from the start, by screening for antigens which successfully induce protection against disease, using a pre-established protocol of immunization. Immunization with attenuated Salmonella can provide both the cell-mediated and antibody-mediated immune responses that will be necessary to successfully vaccinate against plasmodial infection.

In this grant period we expect to develop the attenuated Salmonella system as an expression vector for the cloning of protective plasmodial antigens. Specifically, we expect to identify several recombinant Salmonella which express different P. yoelii blood-stage antigens which can immunize against lethal infection. The final selection of clones will target those antigens which are non-polymorphic and most conserved among Plasmodia. Ultimately, this will facilitate the cloning of the homologous genes from P. falciparum and the construction of a similar attenuated vaccine against the human disease.

III. HBCUs Institutional Characteristics and International Capabilities

Since 1971 Meharry Medical College has operated an International Center for Health Sciences (ICHS). During the seventies the center trained nurse educators from Botswana and other African nations and operated several field stations in African countries to provide on-site training in maternal and child health for African health care workers. From 1985 until 1990 the center participated in a joint memorandum of understanding with Columbia University and A.I.D. to increase Meharry's involvement in A.I.D.'s primary care and child survival activities in developing countries, and in 1987 it became a collaborating center of the World Health Organization, providing community-oriented organization and training for health professionals in developing countries.

Currently, Meharry's principal commitment in international health is to basic research and training in tropical medicine. The objective of our program is to strengthen research capabilities in tropical diseases and to eradicate those diseases which compromise the health and well-being of individuals in developing countries. Table 1 lists A.I.D.-funded projects at Meharry since 1986 which study diseases found in developing countries such as Chagas' disease, sleeping sickness, and vitamin A deficiency. In addition, the college has operated an NIH-funded training program (funded by NIH award 5 T32 AI07281) in the molecular biology of tropical diseases since 1985. The core faculty for this program consists of investigators from Meharry and Vanderbilt University who hold research grants in molecular biology, immunology, and the biochemistry of tropical diseases. The project supports three predoctoral and three postdoctoral trainees. The investigators listed in Table 1 together with faculty and trainees in the training

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program comprise Meharry's Center for Tropical Medicine. Each April since 1984 the Center has sponsored a symposium on tropical diseases featuring scientists at the forefront of research in tropical medicine and parasitology.

Table 1. A.I.D.-funded research on tropical diseases at Meharry since 1986.

Start	End	PI	Title
86/07/01	88/03/31	Hatcher FM	Trypanosome RNA transcription
86/09/29	87/12/31	Valenzuela MS	Replication of trypanosome kinetoplast DNA
86/09/30	87/09/21	Villalta F	T. cruzi membrane components involved in parasite-cell interactions
87/07/31	88/12/31	Villalta F	Role of host iron binding protein Trypanosoma cruzi.....
87/09/30	89/04/30	Valenzuela MS	Characterization of Trypanosoma cruzi kinetoplast networks
88/08/30	89/12/31	Bhorjee JS	Characterization of trypanosome non-histone chromosomal proteins
88.09/30	90/03/31	Villalta F	Trypanosoma cruzi binding to mammalian cells
89/08/01	91/03/31	Valenzuela	Replication of trypanosome kinetoplast DNA; role of minicircle DNA
89/09/29	90/09/28	Villalta F	Trypanosoma cruzi amastigote surface proteins
	91/12/31	Kidane GZ	Molecular cloning of cDNA sequences of L. major
90/08/30	92/07/31	Villalta	Host cell receptors for trypanosoma cruzi
90/08/31	93/02/28	Bhorjee JS	Characterization of histone H1 gene from Trypanosoma cruzi
90/09/25	92/06/30	Hill GC	Purification and cloning of the glycerophosphate oxidase (GPO)
91/08/27	93/08/26	Lima MF	Identification and characterization of host growth factor receptors
91/09/24	93/07/31	Villalta F	Immunobiology of developmentally regulated T. cruzi antigens
92/09/92	94/09/05	Das SK	The effects of vitamin A deficiency on the peripheral benzodiazepine...

It was this commitment to the study of tropical diseases that attracted the P.I. to Meharry as a faculty member in the Department of Microbiology. The present proposal will contribute to the basic research efforts ongoing at Meharry aimed at the improvement of public health in developing countries through the control of tropical diseases. In addition, it will extend these efforts to include malaria, one of the most devastating infectious diseases worldwide.

IV. Collaborative Mechanisms/Networks

The present proposal focuses on the development of a novel strategy for constructing an asexual stage malaria vaccine. The approach may alleviate many of the pitfalls which have been encountered in the pursuit of vaccines against a number of tropical diseases. Research specifically on malaria vaccines is of considerable interest to developing countries and directly relevant to A.I.D's mission. From a broader perspective, the successful completion of this proposal will provide a system that can be applied to other vaccine development efforts including those focusing on other malarias, leishmaniasis, Chagas' disease, toxoplasmosis and mycobacterial infections in particular. By design, this project needs to be conducted in an animal model system and will be carried out entirely in the P.I.'s laboratory at Meharry Medical College.

Dr. Roy Curtiss III (Department of Biology, Washington University, St. Louis) will be involved on this project on a consultant basis. Dr. Curtiss has worked towards the development of

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attenuated Salmonella as vaccine carriers over the past 10 years. He is recognized as one of the leading scientists in this field. Dr. Curtiss will provide the necessary plasmid vectors and attenuated S. typhimurium strains for this project, as well as expert advice concerning the methodologies for the manipulation of the Salmonella system. To facilitate this, the P.I. will spend a brief period of time in Dr. Curtiss' laboratory at Washington University at the beginning of the grant period.

Several opportunities for collaboration with scientists in developing countries are available to the P.I., through previous interactions. During post-doctoral training, the P.I. visited laboratories in Brazil and Colombia and was involved in several projects on leishmaniasis and Chagas' disease with scientists in Brazil and Sudan. Specifically, these included Drs. Roberto Badaro, Edgar Carvalho, Aldina Barral and Manoel Barral-Netto of the Federal University of Bahia (Salvador, Brazil), Dr. Joao Santana da Silva, University of Sao Paulo (Ribeirao Preto, Brazil) and Dr. Hashim Ghalib, University of Juba (Khartoum, Sudan) which led to several publications. These avenues of collaboration are still available to the P.I.. In addition, collaborations are presently being pursued with Dr. William Weidanz (University of Wisconsin-Madison) on a malaria project in Central America. Here at Meharry Medical College, the P.I will be involved in projects with Dr. Donna Russo involving both leishmania and mycobacterial antigens. The present proposal will lay the foundation to expand our efforts to include a number of these investigators, and such collaborations will be pursued as the project develops. Towards that goal we will provide, through these contacts, the opportunity to graduate and post-doctoral students from developing counties to be directly involved in this project from a training perspective.

V. Scientific Aspects of Proposed Work

A. Specific Aims

It is clearly recognized that the development of an effective vaccine against P. falciparum malaria could alleviate one of the most significant public health problems world-wide. Current malaria vaccine efforts have encountered difficulties in identifying protective plasmodial antigens and in delivering these antigens in an appropriate immunogenic form necessary to induce the desired immune response. Over the past 10 years, live attenuated Salmonella strains expressing foreign antigen genes have been developed as safe, stable and inexpensive oral vaccines. Specific antigen genes have been expressed in these Salmonella strains and shown to be capable of inducing protective cell-mediated and antibody-mediated immunity against a number of infectious agents. We propose to use this system to identify, clone and express new plasmodial antigen genes as components of an effective attenuated Salmonella-based malarial vaccine.

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The goals of the proposed research are as follows:

1. To construct a blood-stage P. yoelii cDNA expression library using the balanced lethal host-vector system involving the *cya crp asd* deletion mutant of S. typhimurium (χ4072) and the asd^+ cloning vector, pYA292.

2. To identify plasmodial antigens which can be incorporated into an effective malaria vaccine by

a. screening pools of recombinant Salmonella expressing P. yoelii blood-stage antigens for their ability to immunize mice against a lethal P. yoelii challenge infection.

b. rescreening pools of recombinants that provided complete or partial protection to select Salmonella clones expressing single P. yoelii antigens which can immunize mice against a lethal P. yoelii challenge infection.

c. screening protective Salmonella recombinant clones for their ability to immunize against heterologous challenge with lethal Plasmodium berghei or non-lethal P. yoelii or Plasmodium chabaudi adami parasites.

Due to the selection of recombinants based on their ability to successfully immunize against plasmodial infection, this project must be carried out in an animal model system. This proposal will be limited to this initial phase of the work. A detailed characterization of the structure and function of the selected antigens and a thorough examination of the protective immunity induced will be proposed at a later time. In an attempt to identify conserved, non-polymorphic plasmodial antigens, the final selection of recombinants will be based on their ability to protect against heterologous challenge. This will lay the foundation for the subsequent cloning of the homologous genes from P. falciparum and the construction of a similar attenuated vaccine against the human disease.

B. Rationale

Recent estimates of the global incidence of malaria have risen to 489 million clinical cases per year, with 234 million due to P. falciparum of which 2.3 million result in death (1). In light of the increasing ineffectiveness of current chemotherapeutics and the failure of vector control programs, the desire for the development of an effective malaria vaccine is great (2). Several lines of evidence suggest that the induction of protective immunity to malaria parasites is possible. In children, these parasites produce severe disease and death. As immunity develops with age however, many individuals maintain a low blood parasitemia, but are free of clinical disease (3). The passive transfer of purified immunoglobulin from such immune adults was shown to be capable of reducing parasitemia in children with acute malaria (4). Active immunization from malaria infection was also demonstrated in humans

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with an attenuated sporozoite vaccine (5). Extensive studies in animal models using both immunocompetent and immunodeficient hosts clearly demonstrated that resolution of infection through the natural progression of the disease or through drug therapy rendered animals immune to re-infection. Likewise, immunity to infection has also been induced by immunization with various preparations of crude blood-stage plasmodial antigens. It is clear from these studies that immunity to Plasmodia is quite complex, involving both cell-mediated and antibody-mediated mechanisms (reviewed, 6-8).

Two major approaches to the development of a malaria vaccine have been taken. One has been to identify the targets of the immune response during infection based on the reactivity of sera or T cells obtained following the resolution of infection. This has been difficult particularly for malaria as many of the responses during infection are not protective and in fact may limit the development of protective immunity (8). The alternative approach has been to identify functionally important proteins such as those involved in the invasion of host cells and then target the immune responses to block their activity. With these antigens as with many subunit vaccines, the difficulty has been with their delivery in an appropriate recombinant/synthetic form and context so as to induce a protective and boostable immune response. Several antigens have been identified as vaccine candidates (9,10). A few have been tested or soon will be tested in clinical trials, and it is likely that at least partial protection will be achieved (11-13). Clearly, additional efforts will be required to improve on these first generation subunit malaria vaccines.

The goal of this project is vaccine development and so we will approach the task from an immunization perspective. We feel that it will be difficult to identify the best immunizing antigens based on the immune response of naive individuals who develop acute malaria upon infection. Instead, we will focus on the identification of antigens which promote the induction of protective responses when delivered in a defined vaccine context. The specific context we have chosen is that of a live attenuated Salmonella vaccine carrier. A number of Salmonella strains with a variety of attenuating mutations have been developed as live vaccine strains, capable of expressing foreign antigen genes (reviewed, 14-16). When delivered orally, these bacteria colonize the gut-associated lymphoid tissue and will persist long enough to colonize deeper tissues including the liver and spleen. The result is the induction of strong cell-mediated as well as antibody-mediated immune responses (15); a characteristic which is particularly important for malaria vaccine efforts. These avirulent strains are usually auxotrophic mutants, mutants in regulatory genes or mutants in LPS synthesis and often are not dependent on active immune responses for clearance (16). In fact, strains deficient in aromatic acid compound synthesis have been shown to be avirulent in immunodeficient mice (17,18). Immunization with attenuated Salmonella expressing foreign antigen genes has led to complete or partial protection against a variety of infectious agents including Shigella (19), Streptococci (20), Clostridium (21), Enterotoxigenic Escherichia coli (22), Francisella tularensis (23) ,

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influenza (24), Plasmodia (25) and Leishmania (26). These experiments clearly showed that the immune responses to foreign antigens expressed by the recombinant Salmonella included the induction of CD4+ T cells as well as antibody production, and to a lesser degree the induction of CD8+ T cells.

The attenuated Salmonella vaccine carrier we have chosen is the balanced lethal host-vector system developed by Dr. Roy Curtiss III and colleagues (27-29) involving the *cya crp asd* deletion mutant of S. typhimurium (χ4072) and the *asd*$^+$ cloning vector, pYA292. The attenuation was accomplished by deleting the adenylate cyclase (*cya*) and cAMP receptor protein (*crp*) genes which are required for the transcription of a large number of necessary bacterial genes and operons. This double mutation limits the likelihood of the loss of the avirulent phenotype by reversion or gene transfer, increasing the safety of the vaccine. No change in the virulence of S. typhimurium (χ4072) was observed after repeated in vivo passages (up to 8). Beta-aspartate semialdehyde dehydrogenase (*asd*) is necessary for the biosynthesis of diaminopimelic acid (DAP), an essential cell wall component. Deletion of the *asd* gene allows growth only in the presence of DAP or *asd* complementing plasmids. pYA292 has been engineered to include the S. typhimurium *asd* gene, serving two purposes. One, by selecting recombinants for growth in the absence of DAP, the system eliminates the need for antibiotic resistance markers which are not desirable in live vaccines. Second, the problem of plasmid loss in vivo and thus the loss of expression of the foreign antigen gene is eliminated as the loss of the *asd* gene in the absence of DAP results in cell lysis. With this vector/host combination, 100% of S. typhimurium recombinants retained the expression plasmid in the absence of DAP for over 50 generations in vitro and 3 weeks in vivo. In contrast to systems designed for the stable integration of the expression cassette into the bacterial genome, this system retains the multi-copy feature of the plasmid increasing the potential for higher levels of foreign antigen gene expression. pYA292 also contains a trc promoter, a multiple cloning site in the *lacZα* sequence (allowing for color selection of recombinants based on beta galactosidase activity), a transcriptional terminator from the *rrnB* gene, and a p15A origin of replication. Dr. Curtiss III has recently developed a derivative of pYA292 in which the p15A origin of replication has been replaced with that of pUC. This change should increase the plasmid copy number as much as 100-fold with the goal of significantly increasing the level of antigen expression. We will consider using this high copy number derivative of pYA292, if current experiments evaluating the effectiveness of this vector in vivo prove positive.

We have selected the rodent malaria parasite Plasmodium yoelii for these studies for several reasons. From studies using a number of animal model systems, it is clear that there are antibody-mediated and cell-mediated mechanisms of immunity that can contribute to the resolution of acute malaria infection (6-8). In the P. yoelii model, both arms of the immune response appear to contribute to the development of protective immunity. For example, passive protection

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against P. yoelii can be achieved with polyclonal (30-31) and monoclonal antibodies (32-33) and B-cell deficient mice are unable to resolve their infections (34). Conversely, B-cell deficient mice rescued from P. yoelii infection by drug therapy are protected against challenge infection (34) and the adoptive transfer of CD4+ but not CD8+ T cells from immune mice can confer protection to nonimmune recipients (35,36). It also appears that the Th1 and Th2 subsets of CD4+ T cells may both be important, with the Th1 responses being particularly important early during infection (37,38). From an immunization perspective, it has been demonstrated that mice can be immunized against P. yoelii infection with crude preparations of blood-stage antigens (39,40). In addition, protective P. yoelii antigens appear to be shared among certain species of Plasmodia. P. yoelii immunized mice (39) as well as B-cell deficient mice rescued from P. yoelii infection by drug therapy (34) resist heterologous challenge. Because of these characteristics, we feel that there is a greater potential using the P. yoelii model to apply our results to vaccine development efforts against P. falciparum malaria.

C. Experimental Design

General Methods

Malaria Parasites and Experimental Infections. Plasmodium yoelii 17X (nonlethal) and 17XL (lethal), Plasmodium berghei (ANKA) and Plasmodium chabaudi adami (556KA) were originally obtained from Dr. William P. Weidanz (University of Wisconsin, Madison, WI). All parasites were maintained as cryopreserved stabilates. Adult male C57BL/6 or (BALB/c X C57BL/6)F_1 mice 6 to 10 weeks of age will be purchased from Jackson Laboratories (Bar Harbor. ME) and housed in the animal care facility at Meharry Medical College. This facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Experimental infections will be initiated by intraperitoneal injection of parasitized erythrocytes obtained from donor mice infected with stabilate material. Resulting parasitemias will be monitored by enumerating parasitized RBCs in thin tail-blood smears stained with Giemsa (41).

Specific Aim #1. To construct a blood-stage P. yoelii cDNA expression library using the balanced lethal host-vector system involving the *cya crp asd* deletion mutant of S. typhimurium (χ4072) and the asd^+ cloning vector, pYA292.

Isolation of P. yoelii 17XL poly A(+) RNA (42). Parasitized blood from 50 C57BL/6 mice infected with P. yoelii 17XL will be collected on days 5-7 post-infection, when parasitemias average 40%. As P. yoelii 17XL infection is asynchronous, the parasitized blood will contain a mix of ring, trophozoite and schizont blood-stage parasites. Following centrifugation and removal of the buffy coat of mouse lymphocytes, the pellet of parasitized red cells will be resuspended and passed over columns of microcrystalline cellulose as described (43) to remove remaining mouse leukocytes. This is necessary to minimize the amount of mouse RNA present in the

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preparation of P. yoelii RNA used to construct the library. Parasitized RBCs will be collected and lysed in PBS containing 0.1% glucose and 0.01% saponin. Total RNA will be extracted from the pelleted parasites by the guanidinium/cesium chloride method (44), and poly A(+) RNA isolated by oligo(dT)-cellulose column chromatography (mRNA Purification Kit, Pharmacia).

Construction of P. yoelii cDNA expression library in pYA292. The P. yoelii cDNA library will be constructed in a directionally oriented manner to maximize the number of recombinant clones expressing plasmodial antigens. Using P. yoelii 17XL poly A(+) RNA as template, first strand cDNA synthesis will be primed with a poly(dT) oligonucleotide containing additional 5' sequences to allow the incorporation of a BamHI restriction site. Synthesis will be accomplished with M-MuLV reverse transcriptase in the presence of 5-methyl-dCTP to eliminate the need for the subsequent methylation of BamHI sites. Following treatment with RNAse H, DNA polymerase I and second stand synthesis, adaptors containing an EcoRI 5' overhang will be ligated to either end of the cDNAs. Linkers will be removed by gel filtration and the 5' ends of the cDNAs then phosphorylated. Subsequent digestion of the cDNAs with BamHI will leave EcoRI and BamHI cohesive ends at the 5' and 3' ends respectively, of each cDNA. These cDNAs will be ligated into EcoRI/BamHI digested and dephosphorylated pYA292. This will result in the fusion of the 5' end of each cDNA within the 5' end of the gene for beta-galactosidase increasing the likelihood of an in-frame ligation. The P. yoelii/pYA292 library will be transformed/electroporated into the *cya crp asd* deletion mutant of S. typhimurium (χ4072), and recombinants selected for growth in the absence of diaminopimelic acid and their inability to metabolize X-Gal (blue/white colony formation). Plasmid miniprep DNA will be isolated from 20 recombinants, digested with BamHI and EcoRI, and analyzed by agarose gel electrophoresis to determine the size distribution of the cDNA inserts.

Time-frame : We expect to complete the isolation and purification of P. yoelii 17XL poly A(+) RNA and the construction of the cDNA expression library during the first 2 months of the grant period.

Specific Aim #2. To identify plasmodial antigens which can be incorporated into an effective malaria vaccine by

a. screening pools of recombinant Salmonella expressing P. yoelii blood-stage antigens for their ability to immunize mice against a lethal P. yoelii challenge infection.

The screening of the attenuated Salmonella library of P. yoelii cDNAs for protective antigen genes will be labor intensive and by necessity will initially involve immunization with pools of recombinants. The library will be plated and colonies representing single Salmonella recombinants will be picked and resuspended in 1 ml of L broth (approx. 1 x 10^8 cells/ml) and incubated at 37°C. Each recombinant will be identified by number, and a small amount of the

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resuspended colony will be used to inoculate a master plate for future use and as necessary, for the preparation of frozen glycerol stocks. Following 3-4 hours incubation (density approaching 1 x 10^9 cells/ml), the 1 ml cultures of 100 individual recombinants will be pooled, bacterial cells pelleted and used to orally immunize each of 5 mice (approx. 2 x 10^{10}cells/animal) using an 18-gauge intubation needle. Control mice will be immunized with S. typhimurium containing vector alone. Immediately preceding the oral immunization, animals will receive 0.1 ml of 10% sodium bicarbonate to neutralize gastric secretions and insure maximum survival of inoculum. (BALB/c X C57BL/6)F_1 mice which develop strong immunity to P. yoelii will be used. Previous experiments showed 1) that this strain can be readily immunized against P. yoelii infection (39,40) and 2) that unlike these F_1 mice, the parental strains developed little or no immunity to reinfection with P. yoelii when rendered B-cell deficient (34). Five pools of 100 recombinants each will be screened in each experiment. Three weeks later, mice will be infected i.p. with 1 x 10^4 P. yoelii 17XL parasitized erythrocytes. Parasitemias will be monitored at three time points : 1) the early onset of acute disease (day 4-6); 2) peak parasitemia (day 7-9); 3) following resolution of disease in protected animals (day 10-12). Recombinant pools will be scored for their ability to a) prevent parasitemia, b) prevent mortality or c) delay onset of disease and/or mean day of death with positive results in at least 3 of the 5 animals per group. Peak parasitemia in surviving animals will be considered to compare the effectiveness of immunization among fully or partially protective pools.

Although we will be using pools of recombinants we feel that we will be able to induce a protective response based on two lines of evidence. First, Kumar et al (45) showed that mice could be protected from lethal Plasmodium vinckei infection by co-immunization with a crude preparation of blood-stage antigens and attenuated Salmonella. We feel that it is likely that this crude antigen preparation contained over 100 blood-stage antigens with only a limited number inducing protective responses. Second, protective responses against a number of infectious agents have been induced by immunizations with attenuated Salmonella recombinants using a variety of immunization protocols (19-26). Based on these studies, we limited the number of clones per pool to 100 in order to maintain an immunizing dose of approximately 2 x 10^8 cells of each clone. In addition, we propose to neutralize gastric juices prior to immunization to maximize bacterial survival. This should be an effective dose to confer at a minimum, partial protection.

In each experiment, 500 clones will be tested. We plan to stagger each experiment approximately 2 weeks apart allowing an average of 1000 recombinants to be screened each month. For a plasmodial RNA of relatively low-abundance (0.1% of the total mRNA population (44)), we would need to screen 9000 recombinants to have a 95% probability of detecting a cDNA of that transcript joined in frame to the beta-galactosidase fusion sequence.

Time-frame :We plan to screen 10,000 recombinants over a 1

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year period, months 3 through 14 of the grant period.

b. rescreening pools of recombinants that provided complete or partial protection to select Salmonella clones expressing single P. yoelii antigens which can immunize mice against a lethal P. yoelii challenge infection.

From the 100 pools representing 10,000 recombinant Salmonella, we will select the 4 pools which were most effective in immunizing mice against a lethal P. yoelii 17XL challenge. Each pool of 100 will be broken down and retested in pools of 10 for their ability to provide protection against acute malaria. The final immunizing dose will remain at approximately 2 x 10^{10} cells/animal but the inoculum will now contain approximately 2 x 10^{9} cells of each clone. Control mice will be immunized with S. typhimurium containing vector alone. Immediately preceding the oral immunization, 10% sodium bicarbonate will be administered to neutralize gastric secretions. Infections will be initiated as above and protection assessed using the same criteria as in the primary screen. Due to the decrease in complexity of the recombinant pools and resulting 10-fold increase in the number of cells of each clone per immunization, we expect that the degree of protection observed will be significantly increased.

From the secondary screen, the 4 pools which were again the most effective in immunizing mice against a lethal P. yoelii 17XL challenge will be selected. Each pool of 10 will be broken down and individual recombinant Salmonella clones tested for their ability to immunize against acute malaria. Mice will be immunized in groups of five with an oral inoculum of 2 x 10^{10}cells of each clone per animal. Control mice will be immunized with S. typhimurium containing vector alone. Immediately preceding the oral immunization, 10% sodium bicarbonate will be administered to neutralize gastric secretions. Infections with P. yoelii 17XL will be initiated and protection assessed as above. We expect to be able to identity four protective recombinant Salmonella expressing distinct P. yoelii 17XL blood-stage antigens for further characterization.

Time-frame : We expect to complete the secondary screen over a 4 month period, months 15 through 18 of the grant period. We expect to complete the tertiary screen over a 4 month period, months 19 through 22 of the grant period.

c. screening protective Salmonella recombinant clones for their ability to immunize against heterologous challenge with lethal P. berghei or non-lethal P. yoelii or P. chabaudi adami parasites .

To begin to assess the conservation among various species of Plasmodia of the antigens expressed by the identified P. yoelii recombinants, we will test individually, their ability to immunize against heterologous challenge infection. Following the adminstration of sodium bicarbonate to neutralize gastric secretions as above, each group of five animals will be immunized with one of the four attenuated Salmonella recombinants, by oral administration of 2 x 10^{10} cells/animal. Control mice will be immunized with S.

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typhimurium containing vector alone. Three weeks later mice will be challenge with either lethal P. yoelii 17XL or P. berghei or with nonlethal P. yoelii 17X or P. chabaudi adami. Parasitemias will be monitored as above, with thin tail-blood smears prepared and stained on alternate days beginning 3-4 days post-infection.

Time-frame : We expect to complete the heterologous challenge experiments over a two month period, months 23 and 24 of the grant period.

As discussed earlier, the foremost goal of this project is malaria vaccine development. For a number of reasons (see above), we have taken a novel approach to the problem by predetermining the mode of immunization, namely the delivery of recombinant plasmodial antigens as part of a live attenuated Salmonella vaccine. We then propose to identify the malarial antigens which will be effective in that context. Because of certain limitations of the screening approach to antigen identification, we realize that immunologically relevant parasite antigens may be missed. However, the strengths of the system outweigh this concern and should be reiterated. 1) Since we have chosen to construct a cDNA library, those antigens which are more abundant in the parasite and more likely to boost a primary response, will be represented to a greater degree in the library. 2) Since the approach is dependent on the recombinant expression of plasmodial genes which induce protective immunity to malaria, the selection of recombinants from pools of clones will be skewed towards those antigens which are expressed extremely well in Salmonella and/or are particularly immunogenic upon immunization. 3) The choice of the balanced lethal host-vector system involving the *cya crp asd* deletion mutant of S. typhimurium (χ4072) and the asd^+ cloning vector, pYA292 is of key importance. It is designed to allow the stable, high level expression of foreign genes with 100% of the cells maintaining the expression construct in vivo. This increases the probability that a single recombinant will induce a protective response when immunizations are carried out with pools of clones. 4) The purification of P. yoelii 17XL RNA is designed to avoid any contamination with mouse RNA. The P. yoelii cDNA expression library in Salmonella will be carefully constructed in a directionally oriented manner. These steps will significantly increase the percentage of antigen expressing clones and decrease the number of recombinants that must be screened. These choices make the proposal to screen in vivo for protective constructs feasible and increase the probability that the best malaria vaccine candidate antigens to be delivered as part of a live attenuated Salmonella vaccine will be identified.

In the event that pools of recombinant Salmonella which induce complete or partial protection against lethal P. yoelii infection are not identified in the primary screen of 10,000 clones we will proceed as follows. Mice will be immunized with a combination of P. yoelii crude blood-stage antigens and attenuated S. typhimurium. The immunization protocol will be based on that of Kumar et al (45), in which such co-immunization induced protection against lethal P. vinckei challenge infection. Following the immunization, serum will

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be collected from these animals and parasite specific antibody will be affinity purified on a preparation of immobilized P. yoelii blood-stage antigens (CH-Sepharose). This antiserum will then be used to screen the P. yoelii/S. typhimurium library (20,000 recombinants) by colony immunoblot as described (46), and immunoreactive clones selected. The ability of these clones to immunize against P. yoelii infection will then be evaluated as described above (Specific Aim 2b, 2c and 2d), starting with pools of 5-10 recombinants. Using this approach we will be able to select Salmonella recombinants which are expressing P. yoelii antigens. Since we will base that selection on the reactivity of sera from animals co-immunized with P. yoelii and S. typhimurium, we will again be selecting antigens which are immunogenic in that context. We anticipate that many immunoreactive clones will be identified, and that some of these will likely induce protection against infection during subsequent in vivo evaluation. The disadvantage to this approach is that only those antigens which induce a significant B cell response will be selected. We feel that there will be important antigens which drive a strong T cell mediated protective response that may be missed. Therefore, this strategy will only be used as a secondary approach as the original screening protocol does not impose this selection.

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VI. Compliance with Federal Guidelines and Regulatory Procedures

1. We propose to use a number of species of Plasmodia in this study which cause acute malaria in rodent models. These protozoan parasites infect vertebrates in a species specific manner. Those species intended for use in this application specifically infect rodents and pose no risk of infection to any laboratory personnel.

2. All experiments involving the construction and screening of the P. yoelii 17XL library in attenuated Salmonella host strains will be conducted in accordance with the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

3. Experiments involving the use of laboratory animals will be carried out in a centralized animal care facility in the West Basic Sciences Center of Meharry Medical College. This facility is fully Accredited by the American Association for the Accreditation of Laboratory Animal Care and is responsible for animal care and use in compliance with 1) the U.S. Animal Welfare Act, as amended 1985; 2) NIH Guide for the Care and Use of Laboratory Animals; 3) U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals; and 4) U.S. Government "Principles for the Care and Use of Animals Used in Testing, Research and Education. The animal care facility provides for daily animal care, veterinary care, research services, surgical assistance and monitoring and the purchase of live vertebrate animals. The director of the facility is Dr. Mack Scott. Evidence of registry with the Secretary of Agriculture of the United States is attached.

NIH Office for Protection from Research Risks, Animal Welfare Assurance Number : A3420-01.

VIIa. Qualifications of Principal Investigator

NAME : James M. Burns, Jr. **POSITION** : Assistant Professor

EDUCATION

Delaware Valley College of Science and Agriculture , Doylestown, PA	1984	B.S. Biology
Hahnemann University, Philadelphia, PA	1989	Ph.D. Micro/Immuno
Seatte Biomedical Research Institute, Seattle, WA	1989-1993	Postdoctoral Parasitology

RESEARCH AND PROFESSIONAL EXPERIENCE

1986	Course Assistant, Biology of Parasitism, The Marine Biological Laboratory, Woods Hole, MA
1986 - 1988	Teaching Assistant, Medical Microbiology and Immunology, Hahnemann University School of Medicine, Philadelphia, PA
1988	Lecturer, Medical Microbiology and Immunology (summer session), Hahnemann University School of Medicine, Philadelphia, PA
1989 - 1993	Postdoctoral Scientist, Seattle Biomedical Research Institute, Seattle, WA.
1993-present	Assistant Professor, Meharry Medical College, School of Medicine, Department of Microbiology

PUBLICATIONS

Burns, Jr., J.M., T.M. Daly, A.B. Vaidya, and C.A. Long. 1988. The 3' portion of the gene for a Plasmodium yoelii merozoite surface antigen encodes the epitope recognized by a protective monoclonal antibody. *Proc. Nat. Acad. Sci. (USA)* 85:602.

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Majarian, W.R., T.M. Daly, J.M. Burns, Jr., and C.A. Long. 1988. Plasmodium yoelii: characterization of a protective idiotype during malarial infection in mice. *Experimental Parasitology* 67:227.

Burns, Jr., J.M., L.A. Parke, T.M. Daly, L.A. Cavacini, W.P. Weidanz, and C.A. Long. 1989. A protective monoclonal recognizes a variant specific epitope in the precursor of the major merozoite surface antigen of the rodent malarial parasite Plasmodium yoelii. *J. Immunol.* 142:2835.

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Burns, Jr., J.M., J.M. Scott, D.M. Russo, E.M. Carvalho, C.J. March, K. Van Ness, and S.G. Reed. 1991. Characterization of a membrane antigen of Leishmania amazonensis which stimulates human immune responses. *J. Immunol.* 146:742.

Russo, D.M., J.M. Burns, Jr., E.M. Carvalho, R.J. Armitage, K.H. Grabstein, L. Button, R. McMaster, and S.G. Reed. 1991. Human T cell responses to gp63, a surface antigen of leishmania. *J. Immunol.* 147:3575.

Russo, D.M., S.J. Turco, J.M. Burns, Jr., and S.G. Reed. 1992. Stimulation of human T lymphocytes by Leishmania lipophosphoglycan-associated proteins. *J. Immunol.* 148: 202.

Burns, Jr., J.M., W.G. Shreffler, D.E. Rosman, P.R. Sleath, and S.G. Reed. 1992. Identification and synthesis of a major conserved antigenic epitope of Trypanosoma cruzi. *Proc. Natl. Acad. Sci. (USA)* 89:1239.

Daly, T.M., J.M. Burns, Jr., and C.A. Long. 1992. Comparison of the carboxyl-terminal, cysteine-rich domain of the merozoite surface protein-1 from several isolates of Plasmodium yoelii. *Mol. Bio. Parasitol.* 52:279.

Shreffler, W.G., J.M. Burns, Jr., R. Badaro. H. Ghalib, L.L. Button, R.W. McMaster, and S.G. Reed. 1993. Antibody responses of visceral leishmaniasis patient to gp63, a major surface glycoprotein of Leishmania. *J. Infect. Dis.* 167:426.

Piuvezam, M.R., D.M. Russo, J.M. Burns, Jr., Y.A. Skeiky, K.H. Grabstein, and S.G. Reed. 1993. Characterization of responses of normal human T cells to Trypanosoma cruzi antigens. *J. Immunol.* 150:916.

Burns, Jr., J.M., W.G. Shreffler, D. Benson, H. Ghalib, R. Badaro, and S.G. Reed. 1993. Molecular characterization of a kinesin-related antigen of Leishmania chagasi which detects specific antibody in African and American visceral leishmaniasis patients. *Proc. Natl. Acad. Sci. USA* 90:775.

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CHAPTERS

Melancon, J., J.M. Burns, Jr., A. Vaidya, H.K. Webster, and W.P. Weidanz. 1993. The Immunology of Malaria, in "Immunology and Molecular Biology of Parasitic Infections", 3rd edition, Blackwell Scientific Publications, Inc., Cambridge, MA., pp. 302-351.

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VIIb. Qualifications of Consulting Scientist

NAME : Roy Curtiss III **POSITION** : Professor and Chairman

EDUCATION

Cornell University, Ithaca, NY	1956	B.S. Agriculture
University of Chicago, Chicago, IL	1962	Ph.D. Microbiology

RESEARCH AND PROFESSIONAL EXPERIENCE

1955-1955	Laboratory Instructor and Research Assistant, Dept. of Poultry Husbandry, Cornell Univ., Ithaca NY
1956-1958	Jr. Technical Specialist, Biology Div., Brookhaven Natl. Lab, NY
1963-1972	Biologist, Biol. Div., Oak Ridge Natl Lab, Oak Ridge TN
1969-1972	Leader, Microbial Genetics and Radiation Micro. Group, Biol. Div., Oak Ridge Natl. Lab, Oak Ridge TN
1965-1972	Lecturer, Dept. Micro., Univ of Tenn-Knoxville, Knoxville, TN
1967-1969	Lecturer, UT-Oak Ridge Grad. Sch. of Biomed, Sciences, Oak Ridge TN
1969-1972	Prof.,UT-Oak Ridge Grad. Sch. of Biomed, Sciences, Oak Ridge TN
1970-1971	Assoc. Dir., UT-Oak Ridge Grad. Sch. of Biomed, Sciences, Oak Ridge TN
1971-1972	Interim & Trng Dir., UT-Oak Ridge Grad. Sch. of Biomed, Sciences, Oak Ridge TN
1972-1983	Sr. Scientist, Comprehensive Cancer Ctr., UAB-Birmingham AL
1972-1983	Sr. Scientist, Inst. of Dental Research, UAB-Birmingham AL
1972-1978	Prof. of Microbiology, UAB-Birmingham AL
1973-1982	Dir., Molecular Cell Biology Grad. Program, UAB-Birmingham AL
1975-1983	Prog. Dir., Molecular, Cellular & Medical Genetics Postdoctoral Trng. Prog., UAB-Birmingham AL
1976-1981,1983	Prog. Dir., Infectious Diseases Postdoctoral Trg. Prog., UAB-Birmingham AL
1978-1983	Charles H. McCauley Prof. of Microbiology, UAB-Birmingham AL
1981-1983	Dir, Cystic Fibrosis Research Ctr, UAB-Birmingham AL
1981-1982	Vice Chairman, Department of Microbiology, UAB-Birmingham AL
1982-1983	Acting Chairman, Dept. of Microbiology, UAB-Birmingham AL
1983-1987	Adjunct Professor, Dept. of Microbiology, UAB-Birmingham AL
1983-	Prof. and Chairman, Dept. of Biol., Washington Univ., St. Louis, MO
1983-1991	Prof. of Cellular & Molecular Biol., Sch. of Dental Medicine, Washington Univ., St. Louis, MO
1984-	George William and Irene Koechig Frieburg Prof. of Biology, Dept. of Biology, Washington Univ., St. Louis, MO
1991-	Director, Center for Plant Science and Biotechnology, Washington Univ., St. Louis, MO
1991-	Professor/Collaborator, Iowa State University, Ames, IA

SELECTED PUBLICATIONS

Goldschmidt, R.M., M. Thoren-Gordon, and R. Curtiss III. 1990. Regions of the Streptococcus sobrinus spaA gene encoding major determinants of antigen I. *J. Bacteriol.* 172:3988.

Tinge, S.A., and R. Curtiss III. 1990. Conservation of Salmonella typhimurium virulence plasmid maintenance regions among Salmonella serovars as a basis for plasmid curing. *Infect. Immun.* 58:3084.

Galan, J.E., K. Nakayama, and R. Curtiss III. 1990. Cloning and characterization of the *asd* gene of Salmonella typhimurium: use in stable maintenance of recombinant plasmids in Salmonella vaccine strains. *Gene* 94:29.

Lockman, H.A., and R. Curtiss III. 1990. Occurrence of secondary attenuating mutations in avirulent Salmonella typhimurium vaccine strains. *J. Infect. Dis.* 162:1397.

Curtiss, R. III. 1990. Attenuated Salmonella strains as live vectors for the expression of foreign antigens, pp.161-188. *In* G.C. Woodrow and M.M. Levine (eds.), New Generation Vaccines. Marcel Dekker, Inc., New York.

Curtiss, R. III, and S.M. Michalek. 1990. Vaccines against dental caries due to Streptococcus mutans, pp. 715-740. *In* G.C. Woodrow and M.M. Levine (eds.), New Generation Vaccines. Marcel Dekker, Inc., New York.

Curtiss, R. III, J.E. Galan, K. Nakayama, and S.M. Kelly. 1990. Stabilization of recombinant avirulent vaccine strains in vivo. *Res. Microbiol.* 141:797.

Doggett, T.A., and R. Curtiss III. 1992. Delivery of antigens by recombinant avirulent Salmonella strains. *In* Genetically-engineered vaccines, Ciardi et al., (eds.) Plenum Press. in press.

Gentry-Weeks, C.R., A.L. Hultsch, S.M. Kelley, J.M. Keith, and R. Curtiss III. 1992. Cloning and sequencing of a gene encoding a 21-kilodalton outer membrane protein from Bordetella avium and expression of the gene in Salmonella typhimurium. *J. Bacteriol.* 174:77729.

Jagusztyn-Krynicka, E.K., J.E. Clark-Curtiss, and R. Curtiss III. 1993. Escherichia coli heat-labile toxin subunit B fusions with Streptococcus sobrinus antigens expressed in Salmonella typhimurium oral vaccine strain: importance of the linker for antigenicity and biological activities of the hybrid proteins. *Infect. Immun.* 61:1004.

Tacket, C.O., D. Hone, R. Curtiss III, S. Kelly, G. Losonsky, L. Guers, A.M. Harris, R. Edelman, and M. Levine. 1992. Comparison of the safety and immunogenicity of *aroA aroC* and *cya crp* Salmonella typhi strains in adult volunteers. *Infect. Immun.* 60:536.

Doggett, T.A., E.K. Jagusztyn-Krynicka and R. Curtiss III. 1993. Immune responses to SpaA-expressing recombinant Salmonella. *Infect. Immun.* submitted.

Schodel, F., S.M. Kelley, D.L. Peterson, D.R. Milich, and R. Curtiss III, 1993. Hybrid hepatitis B. virus core/pre-S proteins synthesized in avirulent Salmonella typhimurium and Salmonella typhi for oral vaccination. In preparation.

VIII. Budget Information

The following budget is for a proposed grant period of two years.

Direct Labor	YEAR 1	YEAR 2
a. James M. Burns, Jr, 20% effort		
Principal Investigator, Salary	8,600	8,944
Fringe Benefits	1,720	1,789
b. To Be Named, 50% effort		
Research Assistant I, Salary	10,000	10,400
Fringe benefits	2,000	2,080
PERSONNEL SUBTOTAL		**45,533**
Travel- Domestic		**1,000**
Non-Expendable Equipment		**0**

Other Direct Costs	YEAR 1	YEAR 2
a. Expendable Supplies		
1. Glassware/Plastics (i.e. flasks, pipet tips, tubes, plates, pipets)	2,500	2,500
2. Chemicals (i.e. buffers, culture media, molecular biology reagents)	4,000	2,000
3. Animals		
i. RNA isolation (50 mice)		
Cost: $7.00 x 50 mice	350	
Housing: $0.06 x 50 mice x 15 days	45	
ii. Primary Screen (600 mice)		
Cost: $7.00 x 600 mice	4,200	
Housing: $0.06 x 600 mice x 45 days	1,620	
iii. Secondary Screen (220 mice)		
Cost: $7.00 x 220 mice		1,540
Housing: $0.06 x 220 mice x 45 days		594
iv. Tertiary Screen (220 mice)		
Cost: $7.00 x 220 mice		1,540
Housing: $0.06 x 220 mice x 45 days		594
v. Heterologous Challenge (100 mice)		
Cost: $7.00 x 100 mice		700
Housing: $0.06 x 100 mice x 55 days		330
Animal Subtotal		11,513
SUPPLY SUBTOTAL		**22,513**
b. Publication Costs		**1,000**

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Subgrant/Subcontract Costs	**0**
Cost Sharing	**0**
TOTAL DIRECT COSTS	**70,046**
General and Administrative Costs	**28,789**
TOTAL ESTIMATED COSTS	**98,835**

Budget Justification

James M. Burns, Jr , Assistant Professor, will devote 20% of his time to this project, and proportional salary funds are requested. Dr. Burns has several publications which document his expertise in 1) the construction of genomic and cDNA libraries of Plasmodia, Leishmania and T. cruzi, 2) the screening of these libraries with antibody and DNA probes, 3) the expression and purification of recombinant antigens produced using several bacterial expression systems, and 4) the use of the P. yoelii murine model for the study protective plasmodia antigens. Dr. Burns will directly supervise the entire project and specifically be responsible for the isolation of P. yoelii RNA, the construction of the P. yoelii cDNA expression library, and all immunization and infections. A Research Assistant I will be hired who will devote 50% of his/her time to the project. Specific activities will include the maintenance of parasite stabilates, culture of recombinant Salmonella for immunizations, preparation of thin tail blood-smears from infected animals, staining and reading slides, preparation of frozen stocks of recombinants of interest and recording experimental results. Fringe benefits in the amount of 20% of salary are requested, reflecting current costs at Meharry Medical College. Salary increases in the second year reflect a 4% cost of living adjustment.

Travel funds are requested for the P.I. to attend one national scientific meeting during the second year of the grant in order to present the results of this phase of the project.

Other direct costs include expendable supplies and publication costs. Expendable supplies have been broken down into a) glassware/plastics, b) chemicals, and c) animals. The requested budget was calculated based on expected material usage over the two year period. The larger request for chemicals during the first year reflects the expected costs for molecular biology reagents necessary for the library construction. Greater than 50% of the supply budget is for the purchase and housing of animals. Costs are bases on current lists prices of animals by the Jackson Laboratories, a per diem charge of 6 cents per day per animal in the animal care facility at Meharry, and the expected duration of each experiment. The heart of this proposal centers on developing an in vivo screen for the identification of protective malaria antigens. The number of animals requested is the minimum number that we believe is necessary to obtain a reasonable output of four protective clones, while maintaining a statistically significant number of animals in each group. Funds

requested for publications include costs for manuscript preparation, submission fees, page costs and a minimum number of reprints.

General and administrative costs are calculated at a rate of 41.1% of modified total direct costs in accordance with institutional policy.

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Washington
WASHINGTON·UNIVERSITY·IN·ST·LOUIS

Department of Biology

April 13, 1993

Dr. James M. Burns, Jr.
Assistant Professor
Department of Microbiology
School of Medicine
Meharry Medical College
1005 Eighteenth Avenue, North
Nashville, Tennessee 37208

Dear Dr. Burns,

Thank you for your recent letter inquiring about the possibility of using our recombinant avirulent *Salmonella* antigen delivery systems to explore the identification of potential protective antigens elicited by blood-stage *Plasmodium yoelii*. I am enthusiastic about your approach and have often wondered why more people didn't use the system we have developed as a screen to identify potential protective antigens rather than to assume that some antigen is important and then to go to a lot of trouble to evaluate that idea based on the initial assumption.

I am certainly more than willing to provide you with avirulent *Salmonella* host strains, vectors and advice on how to make constructs. On the other hand, I've overextended myself and don't feel that I can enter into any more collaborative endeavors that require personnel and time commitment of any substantial extent. Nevertheless, we have had people visit our lab for brief periods of time to master some of the initial methodologies and I would be pleased to have you visit in order to accomplish that goal. I might add that although χ4072 has worked well in the hands of some, we have another strain, χ4550 which is related to χ4072 which has done better in the hands of others. We also have made a family of *asd* vectors with differing replicons (pSC101, p15A, pBR and pUC) to afford a range of plasmid copy numbers to thereby influence the amount of antigen expression. Some of these vectors have multiple cloning sites with *Nco*I to facilitate cloning all of the coding sequence with or without a signal sequence whereas others have a diversity of other potential cloning sites. We have also made a series of fusion vectors enabling fusion to LT-B as published in the March issue of Infection & Immunity. In any event, please let me know your needs and I will be happy to supply the appropriate strains and vectors and work out a time for you to visit us to begin to make some of the constructs. Give my best regards to Bob Holt.

Sincerely yours,

Roy Curtiss III

RC/bs

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Campus Box 1137
One Brookings Drive
St. Louis, Missouri 63130-4899
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MEHARRY MEDICAL COLLEGE

DIVISION OF ACADEMIC AFFAIRS
OFFICE OF RESEARCH SUPPORT SERVICES

United States Department of Agriculture Registration

I certify that Meharry Medical College is registered with the Secretary of Agriculture of the United States Department of Agriculture in accordance with the Animal Welfare Act and regulations. The registration number is **63R002** for fiscal year 1993.

Peter J. Dolce, Ph.D.
Director, Research Support Services

4/23/93
Date

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