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CONTRACT NO: DAMD17-87-C-7014

TITLE: PRODUCTION OF ANTIGENS AND ANTIBODIES FOR DIAGNOSIS OF ARBOVIRUS DISEASES

PRINCIPAL INVESTIGATOR: Robert E. Shope, M.D.

CONTRACTING ORGANIZATION: Yale University School of Medicine  
333 Cedar Street  
P.O. Box 20846  
New Haven, Connecticut 06510-8047

REPORT DATE: May 20, 1994

TYPE OF REPORT: Final Report

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SELECTE  
JUN 12 1994  
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PREPARED FOR: U.S. Army Medical Research, Development, Acquisition and Logistics Command (Provisional), Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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94-17975

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DTIC QUALITY INSPECTED 1

94 6 10 082

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

|   |                                      |   |
|---|--------------------------------------|---|
| <b>1. AGENCY USE ONLY (Leave blank)</b> | <b>2. REPORT DATE</b><br>20 May 1994 | <b>3. REPORT TYPE AND DATES COVERED</b><br>Annual Report (4/1/87 - 3/31/91) |
|---|--------------------------------------|---|

|  |   |
|--|---|
| <b>4. TITLE AND SUBTITLE</b><br>Production of Antigens and Antibodies for<br>Diagnosis of Arbovirus Diseases | <b>5. FUNDING NUMBERS</b><br><br>Contract No.<br>DAMD17-87-C-7014 |
|--|---|

|  |  |
|--|--|
| <b>6. AUTHOR(S)</b><br>Robert E. Shope, M.D. |  |
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| <b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b><br>Yale University School of Medicine<br>333 Cedar Street<br>P.O. Box 20846<br>New Haven, Connecticut 06510-8047 | <b>8. PERFORMING ORGANIZATION<br/>REPORT NUMBER</b> |
|--|---|

|  |   |
|--|---|
| <b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b><br>U.S. Army Medical Research, Development,<br>Acquisition and Logistics Command (Provisional),<br>Fort Detrick, Frederick, Maryland 21702-5012 | <b>10. SPONSORING / MONITORING<br/>AGENCY REPORT NUMBER</b> |
|--|---|

**11. SUPPLEMENTARY NOTES**

|   |                               |
|---|-------------------------------|
| <b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b><br><br>Approved for public release;<br>distribution unlimited | <b>12b. DISTRIBUTION CODE</b> |
|---|-------------------------------|

**13. ABSTRACT (Maximum 200 words)**

Reagents were prepared for diagnosis by ELISA of 54 arthropod-borne viruses that cause human disease and are of potential or actual military importance. Mouse brain antigens made by the sucrose-acetone technique to the 54 arboviruses will be useful for assay of arboviral IgG and IgM and may also, in some instances, be used in hemagglutination-inhibition tests. The antigens were inactivated by beta-propiolactone. Rabbit IgG, purified by ammonium sulfate concentration, was prepared to 20 of the arboviruses and was designed to use to capture antigen in ELISA. The titers varied considerably from one rabbit to another, but most may be used in an optimal dilution of 1:1000 to 1:32000.

|   |                            |
|---|----------------------------|
| <b>14. SUBJECT TERMS</b><br>Antigens, Antibodies, Arbovirus, Enzyme linked<br>immunosorbent assay | <b>15. NUMBER OF PAGES</b> |
|   | <b>16. PRICE CODE</b>      |

|  |   |  |  |
|--|---|--|--|
| <b>17. SECURITY CLASSIFICATION<br/>OF REPORT</b><br>Unclassified | <b>18. SECURITY CLASSIFICATION<br/>OF THIS PAGE</b><br>Unclassified | <b>19. SECURITY CLASSIFICATION<br/>OF ABSTRACT</b><br>Unclassified | <b>20. LIMITATION OF ABSTRACT</b><br>Unlimited |
|--|---|--|--|

**SUMMARY**

Reagents were prepared for diagnosis by ELISA of 54 arthropod-borne viruses that cause human disease and are of potential or actual military importance. Mouse brain antigens made by the sucrose-acetone technique to the 54 arboviruses will be useful for assay of arboviral IgG and IgM and may also, in some instances, be used in hemagglutination-inhibition tests. The antigens were inactivated by beta-propiolactone. Rabbit IgG, purified by ammonium sulfate concentration, was prepared to 20 of the arboviruses and was designed to use to capture antigen in ELISA. The titers varied considerably from one rabbit to another, but most may be used in an optimal dilution of 1:1000 to 1:32000.

**FOREWORD**

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

*Robert E. Shope*      *May 20, 1994*  
PI Signature                      Date

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| <b>Accession For</b> |   |
| NTIS                 | GRA&I <input checked="" type="checkbox"/> |
| DTIC TAB             | <input type="checkbox"/>                  |
| Unannounced          | <input type="checkbox"/>                  |
| Justification        |   |
| By                   |   |
| Distribution/        |   |
| Availability Codes   |   |
| Dist.                | Avail and/or<br>Special                   |
| A-1                  |   |

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## BODY OF REPORT

### 1. STATEMENT OF THE PROBLEM UNDER STUDY

The problem under study is the development of ELISA systems for rapid serological diagnosis of human arboviral diseases of military importance. These systems will be applicable in the field for sensitive, specific, and rapid diagnosis of arboviral infections of both civilian and military populations.

### 2. BACKGROUND AND REVIEW OF APPROPRIATE LITERATURE AND/OR EARLIER REPORTS

The enzyme-linked immunosorbent assay (ELISA) was devised by E. Engvall and P. Perlmann in 1971 (Enzyme-linked immunosorbent assay. Quantitative assay of immunoglobulin G. *Immunochemistry* 8:871-874). An antigen or antibody is conjugated to an enzyme allowing quantitative assay when a substrate is added and the reaction is read by color change. The test for the purposes of this project is adapted for detection of an antibody rise in acute and convalescent sera. ELISA is extremely sensitive and does not require the more elaborate equipment needed for the immunofluorescence and radio immune assays. The initial adaptation of ELISA to arboviruses was accomplished at Yale (Frazier, C.L. and Shope, R.E. 1979. Detection of antibodies to alphaviruses by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 11:564-551).

For the detection or capture of antigen, the ELISA requires purified antibody to coat the solid phase and bind the virus. The sensitivity of antibody detection is improved by addition of an initial coating antibody to the ELISA system without increasing background reaction (Yolken, R.H. and Stopa, P.J. 1980. Comparison of seven enzyme immunoassay systems for measurement of cytomegalovirus. *J. Clin. Microbiol.* 11:564-551). In addition it is relatively easy and inexpensive to purify the antibody and relatively more difficult to purify the antigen. Using antibody as coat permits the use of sucrose-acetone processed arboviral antigens which are standard antigens in general use for routine hemagglutination-inhibition and complement fixations tests. Immune rabbit serum has proved satisfactory when purified by ammonium sulfate precipitation. Also, by developing a coating antibody other than mouse (i.e. rabbit) one can use the large number of standardized mouse brain antigens and mouse ascitic fluids available in the reference collections of Yale, CDC, and military laboratories.

### 3. RATIONALE USED IN CURRENT STUDY

The ELISA used in the current study utilized a solid-phase to which was attached semi-purified rabbit anti-arbovirus IgG. This IgG captured homologous arboviral antigen which, in turn, reacted with human or other species antibody. The indicator system was anti-species (e.g. anti-human) antibody to which was conjugated the enzyme, peroxidase. The peroxidase was detected by the substrate ABTS. In order to prepare the rabbit anti-arbovirus IgG, it was necessary first to try to adapt each arbovirus to rabbit tissue culture, RK-13, so that there would be a homologous species of tissue in the immunogen injected into the rabbit. Otherwise, the immune rabbit sera would have been expected to contain antibodies to the heterologous tissue used to immunize.

#### 4. EXPERIMENTAL METHODS

a. The 54 viruses used in the development of the ELISA were as follows:

|                     |                              |                         |
|---------------------|------------------------------|-------------------------|
| Bandia              | Hazara                       | Rocio                   |
| Bangui              | Hughes                       | Ross River              |
| Belterra            | Ilesha                       | Sagiyama                |
| Bhanja              | Ilheus                       | Semliki Forest          |
| Bunyamwera          | Inkoo                        | Sindbis                 |
| Bussuquara          | Jamestown Canyon             | Snowshoe hare           |
| Bwamba              | Japanese encephalitis        | Tahyna                  |
| Cache Valley        | LaCrosse                     | Tataguine               |
| Candiru             | Lymphocytic choriomeningitis | Tensaw                  |
| Catu                | Maguari                      | Tick-borne encephalitis |
| Chagres             | Mayaro                       | Toscana                 |
| Chandipura          | Mucambo                      | VSV-Indiana             |
| Cocal               | O'nyong-nyong                | VSV-New Jersey          |
| Colorado tick fever | Oriboca                      | West Nile               |
| Dugbe               | Oropouche                    | Sicilian sandfly fever  |
| Ganjam              | Piry                         | Zika                    |
| Germiston           | Qalyub                       | Shuni                   |
| Guaroa              | Quaranfil                    | Catu                    |

b. Viruses were passaged intracerebrally in baby mice. When the mice sickened, brains were harvested and antigen was prepared by the sucrose-acetone technique of Clarke and Casals (Clarke, D.H. and Casals, J. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. 7:561-573). Two hundred fifty milliliters of each antigen were produced. The antigens were rehydrated in pH 9.0 tris buffer 0.1M, then infectivity was inactivated by addition of 0.1 % beta-propranolone excepting for the alphaviruses which were inactivated with 0.3 % beta-propranolone. The beta-propranolone/antigen mixture was held for 3 days at 4C, then stored frozen at -70C.

c. Viruses were passaged in a rabbit cell line, RK-13, and observed for cytopathic effect. Those that did not have cytopathic effect were subjected to sub-culture in Vero cells or were tested for antigen content by lysing washed cells and carrying out ELISA by direct coating of the solid phase with the cell lysate. For those that had either infectious virus or antigen (indicating that the virus was replicating), tissue culture fluid was used to immunize rabbits.

d. Rabbits were inoculated with virus in the lateral ear vein at weekly intervals for 3 inoculations. One week later blood was taken and the serum tested by ELISA for its capacity to capture homologous antigen. Rabbits were boosted if needed. When satisfactory titer of antibody was obtained, the rabbits were bled and euthanized. An attempt was made to obtain 300 ml of rabbit serum for each virus. Rabbit sera were subjected to ammonium sulfate precipitation to purify IgG (Hebert, G.A., Pelham, P.L., and Pittman, B. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. Appl. Micro. 25:26-36). Ammonium sulfate treated sera were stored at -20C.

e. ELISA was done by the technique of Meegan et al. (Meegan, J.M., Yedloutschnig, R.J., Peleg, B.A., Shy, J., Peters, C.J., Walker, J.S., and Shope, R.E. 1985. An enzyme immunoassay for detection of antibodies to Rift Valley fever virus in ovine and bovine sera. Am. J. Vet. Research 48:1138-1141).

## 5. RESULTS

### a. Production of antigens:

Inoculation of mice and harvesting of brains for antigen production was completed with the 54 arboviruses. Sucrose-acetone extraction of mouse brains and/or crude brain extraction of the following viruses was completed (liver was extracted in some cases):

| Virus                        | Approximate volume (ml)<br>Sucrose-acetone antigen | Approximate volume<br>crude brains/livers |
|------------------------------|--|---|
| Bandia                       | 284  |   |
| Bangui                       | -  | 150                                       |
| Belterra                     | 239  |   |
| Bhanja                       | 237  |   |
| Bunyamwera                   | 269  |   |
| Bussuquara                   | -  | 78  |
| Bwamba                       | 287  |   |
| Cache Valley                 | -  | 66  |
| Candiru                      | -  | 66  |
| Catu                         | -  | 25  |
| Chagres                      | 97   |   |
| Chandipura                   | 310  |   |
| Cocal                        | -  | 53  |
| Colorado tick fever          | -  | 107                                       |
| Dugbe                        | 259  |   |
| Ganjam                       | 67   | 100                                       |
| Germiston                    | 473  |   |
| Guaroa                       | -  | 133                                       |
| Hazara                       | 266  |   |
| Hughes                       | 280  |   |
| Ilesha                       | 213  |   |
| Ilheus                       | 305  |   |
| Inkoo                        | -  | 125                                       |
| Jamestown Canyon             | -  | 113                                       |
| Japanese encephalitis        | 278  | 27  |
| LaCrosse                     | 13   | 130                                       |
| Lymphocytic choriomeningitis | -  | 100                                       |
| Maguari                      | 268  |   |
| Mayaro                       | 229  |   |
| Mucambo                      | -  | 125                                       |
| O'nyong-nyong                | 375  |   |
| Oropouche                    | 241  |   |
| Oriboca                      | 23   | 60  |
| Piry                         | -  | 30  |
| Qalyub                       | 338  |   |
| Quaranfil                    | 279  | 35  |
| Rocio                        | 87   | 100                                       |
| Ross River                   | 256  |   |
| Sagiyama                     | -  | 125                                       |
| Salehabad                    | 85   |   |
| Semliki Forest               | 182  |   |
| Shuni                        | -  | 73  |

| Virus (continued)       | Approximate volume (ml)<br>sucrose-acetone antigen | Approximate volume<br>crude brains/livers |
|-------------------------|--|---|
| Sicilian sandfly fever  | 37   |   |
| Sindbis                 | 40   | 55  |
| Snowshoe hare           | 306  |   |
| Tahyna                  | -  | 35  |
| Tataguine               | 280  |   |
| Tensaw                  | -  | 125                                       |
| Tick-borne encephalitis | 200  | 37  |
| Toscana                 | 349  |   |
| VSV-Indiana             | 271  |   |
| VSV-New Jersey          | 422  |   |
| West Nile               | 25   | 45  |
| Zika                    | 501  |   |

b. Adaptation of arboviruses to growth in RK-13 rabbit cells:

This was the most challenging aspect of the project from a technical viewpoint. Although initial trials were promising, many of the arboviruses did not replicate, or if they adapted, the tissue culture fluids did not immunize well. The following viruses were satisfactory:

- Bunyamwera
- Bussuquara
- Bwamba
- Chandipura
- Cocal
- Germiston
- Hazara
- Ilheus
- Jamestown Canyon
- Japanese encephalitis
- Mayaro
- Mucambo
- Piry
- Qalyub
- Quaranfil
- Ross River
- Semliki Forest
- Sindbis
- Snowshoe hare
- West Nile

c. Testing of rabbit sera for optimal dilution to use in ELISA:

The ammonium sulfate concentrates of rabbit IgG were tested at several dilutions, usually 1:200, 1:1000, 1:4000, 1:16000, and 1:64000 dilutions. Antigens were used in most cases at 1:10 dilution. There was considerable variation in antibody response of individual rabbits. The results of those antigen-antibody pairs that functioned well are shown below.



| Antigen-antibody      | Antigen dilution | Optimum IgG titer | Volume ml |
|-----------------------|------------------|-------------------|-----------|
| Bussuquara            | 1:25             | 1:200             | 28        |
|                       |                  | 1:400             | 15        |
|                       |                  | 1:2000            | 40        |
|                       |                  | 1:16000           | 34        |
|                       |                  | 1:32000           | 10        |
| Bwamba                | 1:10             | 1:500             | 15        |
| Chandipura            | 1:10             | 1:500             | 27        |
|                       |                  | 1:800             | 47        |
|                       |                  | 1:4000            | 43        |
|                       |                  | 1:32000           | 87        |
| Cocal                 | 1:10             | 1:8000            | 6         |
|                       |                  | 1:16000           | 80        |
|                       |                  | 1:32000           | 96        |
| Germiston             | 1:10             | 1:1000            | 37        |
|                       |                  | 1:16000           | 38        |
| Hazara                | 1:10             | 1:500             | 15        |
|                       |                  | 1:64000           | 42        |
| Ilheus                | 1:10             | 1:64000           | 18        |
| Jamestown Canyon      | 1:10             | 1:16000           | 62        |
|                       |                  | 1:32000           | 30        |
| Japanese encephalitis | 1:10             | 1:1000            | 33        |
|                       |                  | 1:64000           | 13        |
| Mucambo               | 1:10             | 1:400             | 13        |
|                       |                  | 1:500             | 76        |
|                       |                  | 1:4000            | 27        |
|                       |                  | 1:32000           | 40        |
| Quaranfil             | 1:10             | 1:4000            | 85        |
|                       |                  | 1:8000            | 95        |
| Semliki Forest        | 1:10             | 1:32000           | 35        |
|                       |                  | 1:64000           | 40        |
| Sindbis               | 1:10             | 1:4000            | 30        |
|                       |                  | 1:16000           | 30        |
|                       |                  | 1:32000           | 38        |
| Snowshoe hare         | 1:10             | 1:500             | 73        |
| West Nile             | 1:10             | 1:32000           | 15        |
|                       |                  | 1:64000           | 40        |
|                       |                  | 1:128000          | 50        |

## 6. DISCUSSION AND CONCLUSIONS

The major effort of this project was the production of sucrose-acetone extracted mouse brain antigens for 54 viruses. This was for the most part accomplished. Not all of the attempts to immunize rabbits were successful. Those antigens for which a companion rabbit serum is not available will be excellent reagents to use in IgM capture tests of early convalescent sera to make a presumptive diagnosis. They are also highly useful in ELISA with conventional mouse IgG as the capture antibody. Further, many of them can be used in the hemagglutination-inhibition test.

As detailed in the Annual Reports, the project underwent an extensive delay when the only technician, an experienced worker, developed colon cancer. He was away for six months of sick leave and back vacation with full pay. In spite of this, after extensions without additional funds, new personnel were trained and it was possible to a considerable degree to catch up. The final antigens and antibodies should prove to be extremely useful diagnostic reagents.

## 7. PUBLICATIONS

None.

## 8. PERSONNEL RECEIVING CONTRACT SUPPORT

|                         |  |
|-------------------------|--|
| Robert E. Shope, M.D.   | Professor of Epidemiology, P.I.                            |
| James Washington        | Technician A, April 1987 through October 1988              |
| Kaveh Khoshnood         | Associate in Research, July through December 1989          |
| Shirley J. Tirrell, MPH | Associate in Research, January 1990 through<br>March, 1991 |

All financial records pertaining to this contract are subject to audit review.