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STUDIES OF INFECTION AND DISSEMINATION OF RIFT VALLEY FEVER VIRUS IN MOSQUITOES

ANNUAL REPORT

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April 19, 1989

Supported by
U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6133

Ohio University
Athens, Ohio 45701

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: U	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY: U	3. DISTRIBUTION/AVAILABILITY OF REPORT
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE: U	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): NA	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Ohio University; 6b. OFFICE SYMBOL (If applicable):	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Athens, Ohio 45701	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable):	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-C-6133
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62770A	3M16 2770A871	AB	391

11. TITLE (Include Security Classification)

U Studies of Infection and Dissemination of Rift Valley Fever Virus in Mosquitoes

12. PERSONAL AUTHOR(S): William S. Romoser

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 15May87 TO 14May88	1989 April 19	26

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Rift Valley fever; Mosquitoes; Infection; Dissemination; Mosquito Tissue Tropisms; Infection & escape "barriers"; Transovarial Transmission; Electron Microscopy; (over)
06	06		
06	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

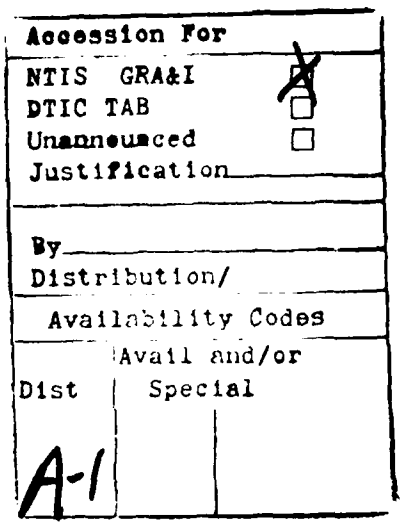
We are engaged in a multimethod study of Rift Valley fever (RVF) virus in vector and hypothetical vector mosquitoes. Major results and conclusions include: (1) The patterns of midgut infection, escape of virus from the midgut, and distribution of virus after entering the hemocoel in Aedes mcintoshi are similar to those in Culex pipiens. (2) Immunocytochemical evidence indicates that the follicular epithelia and eggs of Ae. mcintoshi can become infected by virus in the hemolymph. (3) Out of 1771 Ae. mcintoshi collected from dambos in Kenya, one specimen appeared to be RVF viral antigen-positive by plaque assay. (4) We have made progress in elucidating the histology and ultrastructure of the mosquito proventriculus and the finding of putative virions in the foregut epithelium of specimens with non-disseminated infections supports the idea that RVF virus can enter the foregut epithelium via cells at the foregut-midgut junction. (5) We have found evidence of specific binding of components of formalin-killed RVF virus (vaccine) and mosquito cell membrane preparations.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	U

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

18. (continued) Avidin-biotin-peroxidase complex (ABC) technique; viral plaque assay; vector competence; arbovirus receptor molecules; RAI



SUMMARY

We are engaged in a multimethod study of Rift Valley fever (RVF) virus in vector and hypothetical vector mosquitoes. During this year, we have carried out: (1) an immunocytochemical study of RVF virus in field-collected Aedes mcintoshi, a possible interepizootic RVF virus maintenance species in Kenya; (2) an immunocytochemical study of RVF virus in the male and female reproductive systems of Ae. mcintoshi; (3) an immunocytochemical survey of field-collected Ae. mcintoshi for potential natural infection with RVF virus; (4) preliminary studies of the ultrastructure of the proventriculus of Culex pipiens with regard to possible dissemination of RVF virus via cells at the foregut-midgut junction; and (5) preliminary studies with regard to the mosquito cell surface receptor molecule for RVF virus.

Major results and conclusions from the above studies include: (1) The patterns of midgut infection, escape of virus from the midgut, and distribution of virus after entering the hemocoel in Ae. mcintoshi are similar to those we found earlier in Cx. pipiens and support or are consistent with the conclusions derived from studying the later species. (2) We found immunocytochemical evidence that the follicular epithelia and eggs of Ae. mcintoshi can become infected by virus in the hemolymph. The testicular sheath cells contained antigen in over one-third of the 13 male mosquitoes examined. It is therefore possible that spermatozoa could become infected, but we observed no evidence of this. (3) Out of 1771 Ae. mcintoshi collected from dambos in Kenya, we found one specimen that appeared to be RVF viral antigen-positive, but none of the same 1771 specimens were RVF virus-positive based on plaque assay. (4) We have made progress in elucidating the histology and ultrastructure of the mosquito proventriculus. The finding of putative virions in the foregut epithelium of specimens with non-disseminated infections supports the idea that RVF virus can enter the foregut epithelium via cells at the foregut-midgut junction. (5) Although the search for the mosquito cell surface receptors for RVF virus has just begun, we have found evidence of specific binding of components of formalin-killed RVF virus (vaccine) and mosquito cell membrane preparations.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) 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 (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. Introduction

A. Background

The literature pertinent to this project is extensively reviewed in Hardy et al. (1983), Hardy (1988), Meegan & Bailey (1988), Turell (1988), and in the original research contract proposal.

B. Objectives

The overall objective of this research is to contribute to our understanding of the epidemiology of Rift Valley fever. More specifically, our goals are (1) to describe the dissemination, pathogenesis, and morphogenesis of RVF virus in vector competent and incompetent mosquitoes and (2) to identify the mosquito cell surface molecule (receptor) to which RVF virus binds prior to entering a cell.

Ultimately we hope to shed light on intrinsic factors which influence vector competence using RVF virus/mosquitoes as model systems.

An additional objective is to contribute to studies of the dissemination, tissue tropisms and morphogenesis of selected Nairoviruses and unclassified bunyaviruses in ticks.

C. Research Covered in This Report

In this report, I will summarize the results of (1) an immunocytochemical study of RVF virus in field-collected Aedes mcintoshi, one of the hypothesized interepizootic maintenance species in Kenya (Linthicum et al., 1987); (2) an immunocytochemical study of RVF virus in the male and female reproductive systems of Ae. mcintoshi with comments on the potential for transovarial and venereal transmission of RVF virus in this species; (3) an immunocytochemical survey of field-collected Ae. mcintoshi for potential natural infection with RVF virus; (4) preliminary studies of the ultrastructure of the proventriculus of Culex pipiens with ultrastructural evidence for dissemination of RVF virus via cells at the foregut-midgut junction; and (5) preliminary and equivocal results.

II. Materials and Methods

A. Introductory Comments

We have applied or are planning to apply several methods for localizing viral infection in mosquito tissues. Each method has inherent advantages and disadvantages. Therefore the best approach is to use more than one method (Hardy, et al., 1983). To detect infectious particles in dissected organs and tissues, we are using plaque assay on Vero cells. To detect viral antigen, we are using the avidin-biotin-peroxidase complex (ABC) immunocytochemical technique for light level microscopy (Faran et al., 1986) and are also applying immunocytochemical techniques at the electron microscope level. To detect viral genome, we are involved in the development of a protocol for the application of a peroxidase-labelled complementary DNA probe to paraffin sections of whole mosquitoes. To detect whole virions and nucleocapsids, we are using standard transmission electron microscopy.

The techniques we are applying in our search for the mosquito cell surface receptor for RVF virus include electrophoresis & immunoblotting, enzyme-linked immunosorbant assay (ELISA); affinity chromatography; and experiments involving plaque assay. The ELISA technique is described briefly in Section III.E.1. of this report.

The techniques described below are only those which have been applied and have worked successfully during this year.

B. Plaque Assay

During this year we have used plaque assay primarily to determine the viral "doses" ingested by or injected into mosquitoes used in a given experiment. We have also used plaque assay to determine the dissemination status of a given mosquito.

A brief description of the plaque assay technique follows. Whole mosquitoes and mosquito parts are triturated in 1 mL of mosquito diluent (10% calf serum in Medium 199 with Hank's Salt and antibiotics) and tested for infectious particles by plaque assay on 2- to 4-day-old Vero cell monolayers (Gargan et al., 1983). The mean amount of virus ingested by a sample of mosquitoes taken immediately following each infectious blood meal represents the viral "dose" for a given experiment. In order to determine whether or not virus is present in the body cavity (hemocoel), i.e. whether or not virus has disseminated from the midgut, legs are dissected and assayed.

C. ABC

The ABC technique was developed in 1981 (Hsu, et al., 1981). Faran, et al. (1986) adapted this very sensitive method for use with serial paraffin sections of formaldehyde fixed, whole mosquitoes. The technique is based on the use of primary antibody directed against viral antigen, followed by biotinylated secondary antibody directed against immunoglobulin from the vertebrate in which the primary antibody was formed. Finally, a complex of avidin and biotinylated peroxidase is applied. This complex binds with the biotinylated secondary antibody due to the great affinity between biotin and avidin. The location of the primary antibody/secondary antibody/ABC complex is then rendered visible by the addition of diaminobenzidine tetrahydrochloride (DAB), the oxidative polymerization of which is catalyzed by peroxidase. The DAB polymer appears as a rusty brown precipitate. In our studies the "primary antibody" is actually a blend of monoclonal antibodies directed against RVF virus nucleocapsid protein and two envelope glycoproteins or a monoclonal antibody directed against RVF virus nucleocapsid protein. These antibodies are provided by Cdr. James Meegan at USAMRIID and Dr. Jonathan Smith at USAMRIID respectively.

D. Electron Microscopy

To prepare mosquito tissues for electron microscopy, tissues are (1) fixed in Karnovsky's solution (1.5% glutaraldehyde, 2.0 % formaldehyde in 0.1M phosphate buffer and 0.15M sucrose) for 1 1/2 to 2 hrs. at 0-4^{o}C; (2) placed in buffered sucrose (15 min. to 48 hours); (3) post-fixed in 1.0% buffered osmium at 0-4^{o}C for 1 hr.; (4) dehydrated by passing through an ethyl alcohol series; (5) placed in propylene oxide for 20 min., then 1:1 volumes of propylene oxide and resin (Epon 812; Araldite 502; DDSA; DMP-30) for 1 hr., the 1.0 mL of resin was added; (6) 3-24 hours later, tissues are embedded in aluminum foil pans and placed in an oven at 60^{o}C for 48 hrs. Blocks of embedded tissues are cut on an ultramicrotome using a diamond knife, mounted on copper grids, and stained with uranyl acetate and lead citrate. Specimen grids are studied and electron micrographs taken with a Zeiss 110 transmission electron microscope.

C. Specific Investigations

1. Immunocytochemical Study of RVF Virus in Ae. mcintoshi

A large number of female Ae. mcintoshi (collected during May & June, 1986 from an artificially flooded dambo at Sakari Ranch just outside of Nairobi, Kenya) were allowed to ingest blood from a viremic hamster. The amount of virus ingested ranged from $10^{6.7}$ to $10^{6.9}$ plaque forming units (PFU) of virus. Subsequently, samples were killed and fixed in 5% formaldehyde 3, 5, 7, 8, 14, 15 and 21 days following the infectious blood meal. Specimens were incubated at 26°C. Following fixation, specimens were stored in 70% ethyl alcohol. In addition to the specimens given a viremic blood meal, several female mosquitoes were intrathoracically (IT) inoculated with virus incubated for several days and fixed and stored as above. All specimens were then prepared for light microscopic examination by application of standard microtechnical procedured followed by the ABC complex immunocytochemical technique.

2. Immunocytochemical Study of RVF Virus in the Female and Male Reproductive Systems of Ae. mcintoshi

The procedures described below were carried out on specimens of Ae. mcintoshi collected from a flooded dambo at Sakari Ranch outside Nairobi in May, 1987. Our goal was to see if, and to what extent, the female reproductive tissues become infected by RVF virus from the hemocoel after at least two blood meals.

Several female mosquitoes were IT inoculated with a strain of RVF virus from Kenya (C3/36--6/12/86) and incubated at 26°C. After 3 days incubation, specimens were allowed to ingest a blood meal, digest and assimilate this meal, and then to oviposit. Specimens were then given a second blood meal and incubated for about 3 days to provide time for vitellogenesis. Gravid females were then killed and fixed in 5% formaldehyde for approximately 5 hours and stored in 70% ethyl alcohol.

Several male mosquitoes were IT inoculated with RVF virus (Kenyan strain C6/36--6/12/86) and a sample injected with C6/36 diluent (negative controls for the ABC procedure). Specimens were incubated at 26°C for periods of time varying from 6-13 days, fixed in 5% formaldehyde and stored in 70% ethyl alcohol.

Serial paraffin sections were prepared and the ABC immunocytochemical procedure was appplied to all specimens.

3. Immmunocytochemical Survey of Ae. mcintoshi for Natural Infection

Our objective in this study was to survey a relatively large sample of field-collected mosquitoes for natural infection with RVF virus using standard plaque assay on Vero Cells and the ABC procedure on each mosquito collected. This allowed us to see is the techniques would both detect the same infection and provided the possibility of detecting the histological location of a natural infection.

A total of 1,771 mosquitoes were collected in May, 1987 from dambos at Sakari Ranch outside of Nairobi. Sexes, numbers, stage of mosquitoes at the time of collection, location of collection and initial treatment were as follows: females collected as pupae (and allowed to emerge before fixation) from an artificially flooded dambo, 173; females collected as adults in region of dambos, 99; males collected as adults, 539; males and females collected as pupae from dambos, allowed to emerge as adults, and frozen, 960.

All specimens were prepared for application of the ABC procedure & microscopic examination and for plaque assay. The females collected as pupae and allowed to emerge as adults were given 3 different opportunities to ingest blood from uninfected hamsters. In all cases, body parts of each specimen were divided into two portions, one portion frozen for later plaque assay and one portion fixed in 5% formaldehyde and stored in 70% ethyl alcohol for later preparation of serial paraffin sections, application of the ABC complex procedure and light microscopic examination. From live mosquitoes, the abdomens were frozen for plaque assay and the intact heads and thoraces prepared for immunocytochemical examination. From frozen mosquitoes, specimens were prepared identically to live mosquitoes, or heads and legs were frozen for plaque assay and the intact thorax and abdomen prepared for immunocytochemical examination.

In an effort to be as efficient as possible in the application of the ABC complex procedure, samples of near mid-saggital sections from 5 specimens were mounted on a single teflon-ringed immunocytochemistry slide. This enabled us to include 90 specimens, along with appropriate controls, in a single run of the ABC technique. For each ABC complex run, the following controls were used: IT inoculated Cx. pipiens, El Gabal strain, treated in a fashion identical to the "unknowns", positive control (to indicate whether or not a positive specimen among the "unknowns" would be detectable); diluent-injected Cx. pipiens, negative control treated in a fashion identical to

the "unknowns" (to check for non-specific reactions, i.e. false positives); IT-infected Cx. pipiens to which a different mouse immunoglobulin G (anti-sarcoma 180) was used in place of the primary antibody (a "blend" of mouse anti-RVF virus monoclonal antibodies). The last control was included to check for the possibility that RVF viral infection causes mouse immunoglobulin G to bind non-specifically to mosquito tissues).

4. Ultrastructural Studies of the Proventriculus of Cx. pipiens & Evidence for Dissemination of RVF virus via Cells at the Foregut-midgut Junction.

Many specimens of Cx. pipiens females, blood fed & non-blood fed (from viremic hamsters or pledget) and RVF virus-infected and non-infected, were prepared for electron microscopic examination as described in II.D. of this report.

III. Results & Discussion

A. Immunocytochemical Study of RVF Virus in Ae. mcintoshi

1. Infection and dissemination rates

Thirty-eight of 63 or 60.3% of the specimens we studied became infected (Table 1). Mean daily infection rate was 64.3 ± 26.4% (range = 0 - 100). This overall infection rate in Ae. mcintoshi is somewhat lower than found in Cx. pipiens (81.8%) which ingested an essentially identical amount of virus (ZH501 strain; Annual Report #1). The overall dissemination rate (based on viral titration of legs and/or immunocytochemical examination) was 29 out of 38 infected specimens or 76.32% (Table 2). The mean daily dissemination rate was 80.2 ± 21.8% (range = 20.0 - 80.0%). Among infected individuals, Ae. mcintoshi appears to show a greater dissemination rate than Cx. pipiens (48.3%; Annual Report #1). However, it should be noted that a different strain of RVF virus was ingested by Ae. mcintoshi.

2. Patterns of midgut infection & escape of virus from the midgut

As with Cx. pipiens, RVF antigen was detected in all midgut regions of Ae. mcintoshi, i.e. the cardial epithelium; the tubular, anterior midgut; and the baglike, posterior midgut. Table 3 shows the relative frequency of infection of each of the 3 regions mentioned. Infections tended to be somewhat localized as opposed to covering broad areas. In contrast with Cx. pipiens, the frequency of infection of the cardial epithelium and tne posterior midgut was considerably lower in Ae. mcintoshi, 27.3% versus 49.1%

Table 1

Infection based on the presence of detectable RVF viral antigen in the intussuscepted foregut and/or midgut.

Days after Infective Blood meal	Sample Size	Number Infected*	Percent Infected
2	10	0	0
3	10	8	80
5	10	7	70
7	10	5	50
8	10	8	80
14	10	5	50
15	10	2	20
21	3	3	100
Total	63		

Table 2

Dissemination based on the presence of infective RVF virus the legs (plaque assay) or antigen in the intussuscepted foregut or in tissues on the hemocoel-side of the midgut.

Days after Infective Blood meal	Number Infected	Number with Disseminated Infections	Percent of Infected Specimens with Disseminated Infections
3	8/10	7	87.5
5	7/10	4	57.1
7	5/10	5	100.0
8	8/10	4	50.0
14	5/10	5	100.0
15	2/10	2	100.0
21	3/3	2	66.7
Total	38/63	29	

Table 3

Distribution of RVF viral antigen in the midguts of orally infected mosquitoes.

Organ	RVF viral antigen-positive*
Cardial Epithelium	27.3 (9/33)
Anterior Midgut	75.8 (25/33)
Posterior Midgut	51.5 (17/33)

*Percent positive for RVF viral antigen (number positive/total number examined)

and 51.5% versus 83.3% respectively. However, again, different viral strains were involved.

Virus escape from the midgut has been assumed to occur via the midgut epithelial cells, especially in the posterior midgut. However, as suggested by Romoser et al. (1987) virus may also disseminate via cells at the foregut/midgut junction and into the intussuscepted foregut (Figs. 1 & 2, IF). Following this route, virus would infect the IF and associated sphincter muscle, then the hemolymph and/or the tissues of the diverticula/esophagous region, and finally the hemolymph Romoser et al., 1987).

In Ae. mcintoshi patterns of RVF antigen distribution in the midgut and IF in association with information on dissemination status are consistent with the pattern found in Cx. pipiens (Romoser et al., 1987) and support the hypothesis of virus escape via the IF (Table 4). Two individuals with disseminated infections were found with antigen only in the IF, indicating that this region can be infected with virus from the gut lumen and that dissemination can occur from this site.

Again as suggested by the study of Cx. pipiens, dissemination of RVF virus via the IF may be a very common occurrence (Table 4). Most individuals, 14 out of 20 or 70.0% of individuals with non-disseminated infections had antigen in the anterior and/or posterior midgut, but not in the IF. On the other hand, all individuals, 9 out of 9, with disseminated infections had antigen in the IF.

On the other hand, several Ae. mcintoshi with disseminated infections in which the anterior and/or posterior midgut were infected, but not the IF (Table 4), indicating that virus also escapes via the midgut epithelial cells.

3. Distribution of virus following escape of virus from the midgut

As with our study of Cx. pipiens (Annual Report #1), several tissues (foregut, including the intussuscepted foregut; fat body; ganglia; salivary glands; epidermis; and ommatidia of the compound eyes) were selected and examined in order to evaluate the dynamics of dissemination of RVF virus from the midgut. Data pertinent to these tissues were examined in two ways: (1) calculation of a daily "dissemination index" (DI) for mosquitoes with disseminated infections for each day studied following an infective blood meal; and (2) accumulative percent infection as a function of time following an infective blood meal. The DI provided a way to estimate the relative extent of infection (antigen distribution) in a given individual with a disseminated infection.

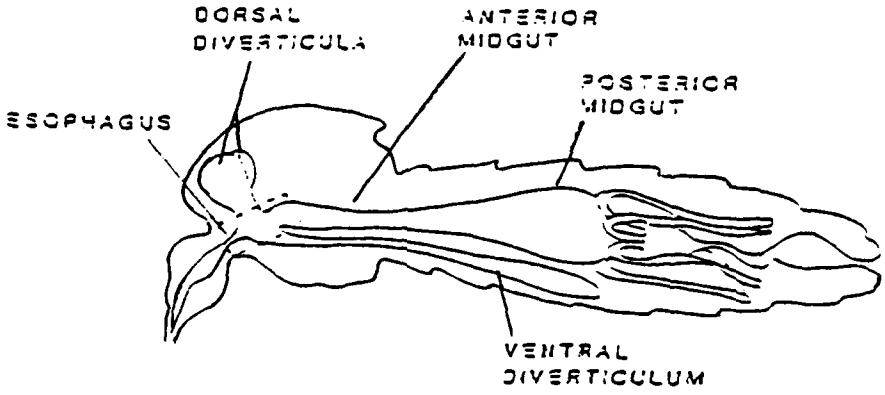

Figure 1. Mosquito alimentary canal

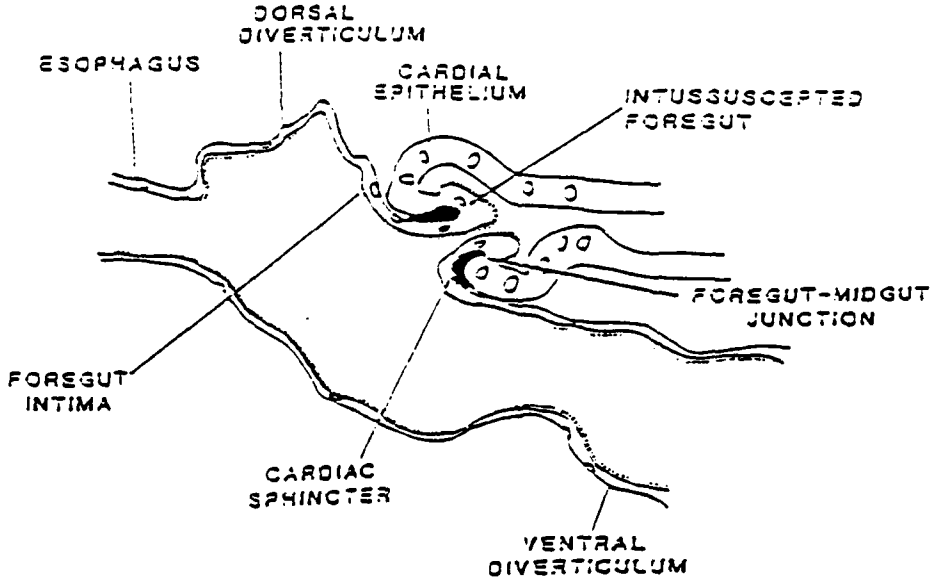

Figure 2. Sagittal section in region of the foregut-midgut junction (indicated by boxed area in Fig. 1). The esophagous, dorsal diverticulum, ventral diverticulum, and intussuscepted foregut are all foregut tissues.

Table 4

Patterns of RVF viral antigen distribution in the midgut and intussuscepted foregut of Culex pipiens orally exposed to RVF virus.

Antigen-positive region(s)*	% Nondisseminated (no. obs.)	% Disseminated (no. obs.)
1. IF only	0	10.0 (2)
2. IF/Ant. Mg.	0	25.0 (5)
3. IF/Post. Mg.	0	0 (0)
4. IF/Ant. Mg./Post. Mg.	0	35.0 (7)
5. Ant. Mg. only	33.3 (3)	10.0 (2)
6. Ant. Mg./Post. Mg.	44.4 (4)	10.0 (2)
7. Post. Mg. only	22.2 (2)	10.0 (2)
Total	100.0 (9)	100.0 (20)

*IF, intussuscepted foregut; Ant. Mg., anterior midgut; Post. Mg., posterior midgut.

In Ae. mcintoshi, the DI was determined for each day following the infective blood meal by examining the following 9 tissues to determine how many had at least some RVF viral antigen-positive cells: salivary glands; ommatidia of the compound eyes; foregut; fat body in the head, in the thorax and in the abdomen; ganglia in the head, in the thorax, and in the abdomen; and finally epidermis in the head, in the thorax, and in the abdomen. The number of tissues which were antigen-positive were summated and divided by 9 to give the dissemination index. An index of 1.00 indicated that all tissues were antigen positive in a given specimen. An index of zero indicated that dissemination had not occurred (at least on the basis of antigen distribution). It should be noted that we determined the dissemination index for all individuals which had disseminated infections on the basis of the presence of antigen or the presence of infectious virus in the legs.

Figure 3 presents the dissemination index for each day following the infective blood meal in all individuals with disseminated infections. The extent of dissemination on any given day examined varies considerably and there are almost no intermediate values. These results are essentially identical with those from our study of Cx. pipiens and our conclusions from that study are thereby strengthened: "Since there are instances where the dissemination index is very low, even as late as day 12, these results appear to indicate that dissemination of RVF virus from the midgut a occurs sporadically over a long period of time. The dearth of intermediate values is consistent with findings based on plaque assay of dissected organs and tissues that once RVF virus disseminates beyond the midgut, it rapidly infects the tissues in the hemocoel." (Annual Report #1).

As we reasoned earlier (Annual Report #1), since dissemination occurs sporadically, the extent of dissemination among any given day's sample would be expected to range from individuals in which virus has just left the midgut to those in which virus has been present in the hemocoel for a long time. At any given time after an infectious blood meal, the tissues "outside" of the midgut which tend to become infected first would be expected to have the highest frequency of infection, and the last tissues to become infected would be expected to have the lowest frequency of infection. The validity of our argument hinges upon whether or not all tissues used to determine the DI are equally susceptible to infection. Such is the case in both Cx. pipiens and Ae. mcintoshi since the tissues used to determine the DI become 95-100 % infected in intrathoracically inoculated individuals which have been incubated for several days.

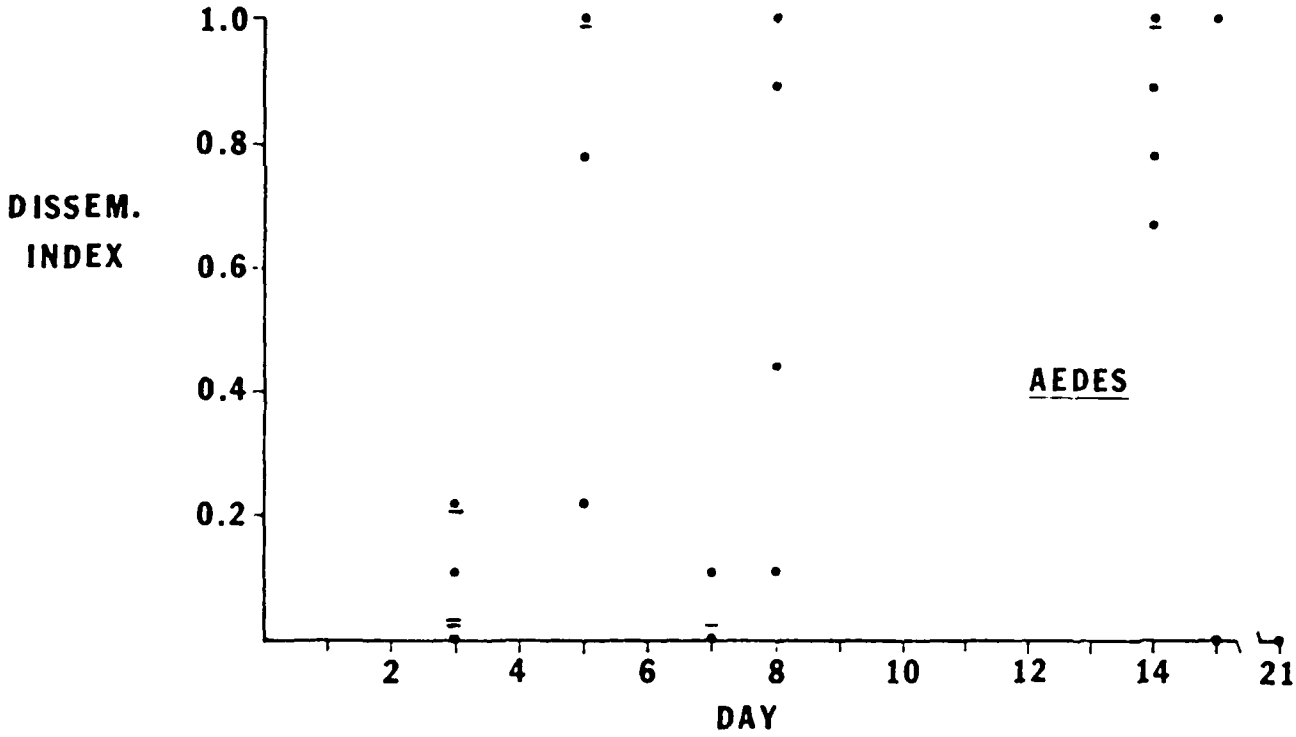

Figure 3. Dissemination index and time following an infective blood meal in Aedes mcintoshi

Display of the relative frequency of infection of various tissues and organs upon which the DI is based should provide information regarding the sequence of infection.

Figure 4 presents the accumulative percent infection over time and facilitates comparison among the tissues over time. There is a consistent pattern, with the infection frequency of fat body and IF being considerably higher than the other tissues and organs. Figure 4 implies that the fat body and IF become infected consistently earlier than the other tissues.

Applying the reasoning outlined above, Figures 4 and 5 suggest that the fat body and IF are infected first, and then the remaining tissues are infected. These results are not inconsistent with the IF as a route of virus escape from the midgut lumen and into the hemocoel. However, in Cx. pipiens the frequency of IF infection was consistently higher than in Ae. mcintoshi. As was evident in Cx. pipiens and is evident here, the extensive infection of the fat body suggests that this massive tissue serves as a major amplifying tissue as earlier suggested by Weaver (1986). The IF and foregut tissues would also appear to play such a virus amplifying role. A relative similarity of infection frequency between the salivary glands, ganglia, epidermis and ommatidia was found in Cx. pipiens and is the case here, suggesting that once virus escapes from the midgut and is amplified by the IF and/or fat body, it spreads to the other tissues rapidly.

It seems a good possibility that in cases where different arboviruses disseminate sporadically in their mosquito hosts, our approach to data analysis will be of value in comparing the dynamics of infection.

The RVF virus mosquito tissue tropisms we have found in Ae. mcintoshi are essentially identical to those we found in Cx. pipiens and therefore needless repitition of data tables will be avoided here. It seems safe to state that RVF virus is pantropic in the mosquitoes studied to date, indicating that this virus could negatively affect both energy reserves as well as regulatory functions in mosquitoes.

B. Immunocytochemical Study of RVF Virus in the Male and Female Reproductive Systems of Ae. mcintoshi

The results of light microscopic immunocytochemical study of the reproductive system of 35 female Ae. mcintoshi are shown in Table 5. These mosquitoes had been IT infected with RVF virus, provided with more than one blood meal, and allowed to oviposit at least once. The data presented represent only specimens in which chorionated eggs were present.

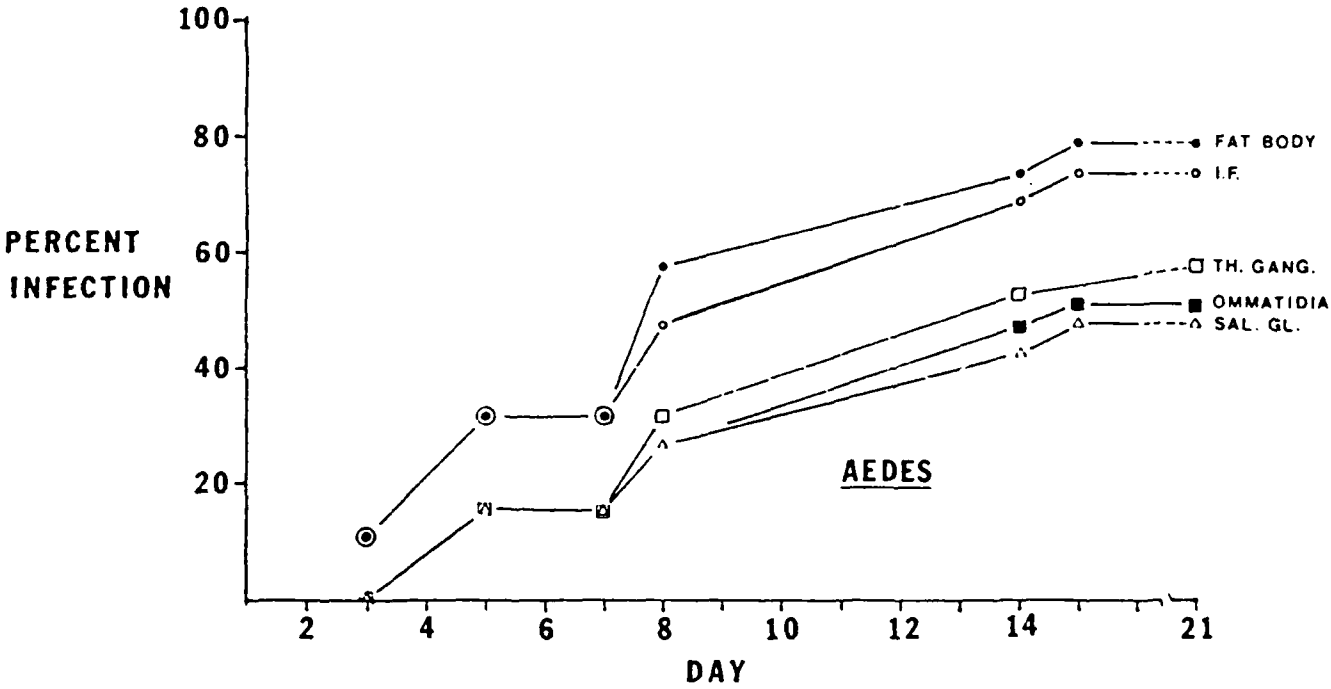

Figure 4. Accumulative percent infection of selected tissues and organs following an infective blood meal in Aedes mcintoshi

Table 5

Distribution of RVF viral antigen in the female reproductive system of Aedes mcintoshi*

Tissue/Structure	No. RVFV Antigen +/ No. Observed (% Ag. +)
Ovarian Sheath	26/35 (74.3)
Ovariole Sheath	18/35 (51.4)
Germarium	0/35 (0)
Penultimate Follicle:	
Epithelium	1/35 (2.9)
Oocyte/nurse cells	1/35 (2.9)
Ultimate Follicle:	
Oocyte/nurse cells	3/35 (8.6)
epithelium-anterior	22/35 (62.9)
epithelium-central	19/34 (55.9)
epithelium-posterior (pedicel/calyx)	27/32 (84.4)
Lateral Oviduct	32/35 (91.4)
Common Oviduct	31/34 (91.2)
Genital Chamber	26/33 (78.8)
Cells near spermathecal pores	20/31 (64.5)
Spermatozoa	0/23 (0)
Accessory Gland	0/25 (0)

* Specimens intrathoracically infected with RVF virus, provided with more than one blood meal (before or after IT infection), and allowed to oviposit at least once. The data presented represent only specimens with chorionated eggs.

Both ovarian sheaths and ovariole sheaths were observed to be infected in many individuals, 74.3% and 51.4% respectively. In no case was the germarium observed to be infected. A single penultimate follicle was observed to be infected, including the follicular epithelium and the oocyte/nurse cells. Among the ultimate follicles which contained chorionated eggs, RVF viral antigen was seen in a single instance in three different specimens, i.e. 3/35 or 8.6%. A much higher frequency of infection was observed in the follicular epithelium of ultimate follicles, 62.9% in the cells associated with the anterior part an egg, 55.9% in the cells of the central region of an egg, and 84.4% in the cells associated with the posterior region of an egg (including the adjacent pedicel and calyx. The oviducts and genital chamber all displayed high frequencies of infection as did cells near the spermathecal gland ducts, the tiny ducts that open into the lumen of a spermatheca. In no cases were the accessory gland (caecus) or spermatozoa in the spermatheca seen to be infected.

Based on our results, the following comments and conclusions seem warranted: (1) Eggs of Ae. mcintoshi can apparently become infected with RVF virus from the hemocoel. It thus seems possible that a new transovarially transmitting line of mosquitoes could become established by a female mosquito feeding on a viremic host. (2) Since the ovarian and ovariole sheaths and follicular epithelia contain antigen, RVF virus probably gains access to follicles via these cells. (3) It appears that the germarium does not become infected with virus from the hemocoel. However, it seems possible that this tissue could be infected transovarially if primordial germ tissues were infected during the larval or early pupal stages. (4) Since the calyx and lateral oviducts contain antigen, it is conceivable that virus is shed into these areas and then gains entry to eggs directly via the micropyle or indirectly via the spermatozoa at fertilization. (5) Since cells near the spermathecal gland ducts (putative spermathecal gland cells) often contain antigen, it is intriguing to consider that virus might gain entry to the spermathecae via these ducts and infect or adsorb onto spermatozoa or pass down the spermathecal ducts and enter the lumen of the genital chamber. Although we have not seen RVF viral antigen-positive spermatozoa, it is possible that virus either gains entry, but does'nt replicate or that virus attaches to surfaces of spermatozoa and "rides piggyback" to an egg, entering the egg through the micropyle along with the spermatozoa. (6) Since the accessory gland was not seen to contain antigen, this potential route to the genital chamber and then to eggs via micropyles or spermatozoa seems unlikely. (7) Tracheae are difficult to interpret immunocytochemically, but could represent a route of ingress of virus into ovarian tissues. Electron microscopy and/or use of a cDNA probe for in situ study may clarify this

possibility. (8) Although 4 individual eggs infected in 4 different mosquitoes may seem to be a low frequency of infection, the fact that 4 our of 35 individuals (11.4%) contain any RVF antigen-positive eggs is significant.

The results of immunocytochemical examination of the male reproductive system in Ae. mcintoshi are shown in Table 6. Although the fat body investing the testes was invariably infected with RVF virus, the sheath cells of the testes were infected in only 38.5% of the specimens and the cells within the testes were all negative for RVF viral antigen. Other than one instance where antigen was observed in cells of the vas deferens, no other male reproductive tissues, including spermatozoa were seen to contain antigen.

Since the testicular sheath cells were observed to be infected in over one-third of the specimens examined, it is possible that given longer incubation times the testicular cells would have become infected, possibly leading to infected spermatozoa. It is also possible that spermatozoa could be infected, but that no viral replication occurs and hence virus is not detectable immunocyochemically.

C. Immunocytochemical Survey of Ae. mcintoshi for Natural RVF Virus Infection

We have examined "survey slides" representing 1771 specimens of Ae. mcintoshi collected from flooded dambos in Kenya and have found one putative RVF virus antigen-positive specimen. Apparent antigen-positive cells appear to be located in the hindgut epithelium and are evident in serial sections. Since this specimen was collected as a pupa the presence of antigen would indicate both a natural infection and the occurrence of vertical transmission. No RVF virus-positive specimens have been found as the result of plaque assay, including the single putative antigen-positive specimen.

D. Ultrastructural Study of the Proventriculus of Cx. pipiens and Evidence for Dissemination of RVF Virus Via Cells at the Foregut-midgut Junction

The proventriculus forms as an intussusception of foregut and midgut epithelia. The outer layer is midgut tissue (the cardial epithelium). Inner layers consist of esophageal epithelium which enters the cavity formed by the cardial epithelium and turns ectad and then anteriorly, forming the reflected wall. The adult foregut intima ends on the reflected wall of the esophagous. The midgut basal lamina enters the proventriculus as the cardial epithelial cells turn entad, appearing as a distinct, thin, moderately electron dense line. However, within the proventriculus, just behind the bases of the cells of the reflected wall of the esophagous, it fades into the loose, "spongy" layer

Table 6

Distribution of RVF viral antigen in the male reproductive system in intrathoracically infected Aedes mcintoshi

Tissue/Structure	No. RVFV Antigen +/ No. Observed (% Ag. +)
Fat body investing testes	6/6 (100.0)
Testicular sheath	5/13 (38.5)
Testicular cells	0/11 (0)
Vas efferens (including contents)	0/6 (0)
Vas deferens (including contents)	1/6 (16.7)
Seminal vesicles	0/12 (0)
Accessory gland (including contents)	0/14 (0)
Ejaculatory duct (including contents)	0/9 (0)

(matrix; King, 1988) which occurs between the entad and ectad layers of the esophageal epithelium. Both longitudinal and circular muscle fibers are apparent between the esophageal walls, implying the ability to act as a sphincter and to move the walls of esophageal epithelium anteriorly and posteriorly within the cardial epithelium.

We have found putative RVF virions in the intussuscepted foregut within the adult proventriculus in specimens with non-disseminated infections on the basis of plaque assay of dissected legs. This supports the occurrence of infection of the cells at the foregut-midgut junction from the midgut lumen as mentioned earlier in this report and in Romoser et al. (1987).

E. Preliminary & Equivocal Results

1. Mosquito Cell Surface Receptor for RVF Virus

Our search for the mosquito cell surface receptor for RVF virus has only recently begun and no definitive results are available for this report. However, we have found evidence of specific binding of components of formalin-killed RVF virus (vaccine) and mosquito cell membrane preparations. The following abstract submitted for presentation at the 1988 meeting of the American Society of Tropical Medicine and Hygiene summarizes our preliminary findings:

EVIDENCE FOR THE SPECIFIC BINDING OF RIFT VALLEY FEVER VIRUS TO COMPONENTS OF SOLUBILIZED MOSQUITO TISSUES AND CULTURED CELLS (Adel A. Mikhail, Maria Lozykowski & William S. Romoser)

"In an effort to isolate possible cell surface receptor molecules for Rift Valley fever (RVF) virus we have developed an enzyme-linked immunosorbent assay (ELISA) to test for the presence of specific virus-binding molecules in extracts of mosquito tissue.

Microtiter plates were coated with cell membrane preparations from homogenized whole adult mosquitoes or cultured Aedes albopictus (C6/36) cells and incubated overnight. After conventional blocking, RVF vaccine (formalin-killed virus) diluted with PBS was applied and incubated an additional 12 hours. The plates were washed vigorously and the presence of bound virions was detected by the addition of a mixture of monoclonal antibodies directed against RVF virus envelope glycoproteins, G_1 and G_2, and against nucleocapsid protein. Subsequently, a conjugate of HRP and goat anti-mouse antibody was added. After reaction with an appropriate substrate, optical density was determined with an ELISA reader.

Using the above assay we have found evidence of specific binding of RVF virus to a component or components

of both whole body and cultured cell homogenates. Further, this binding is diminished upon treatment with 2-mercaptoethanol. We view this specific binding as a possible indicationof the presence of mosquito cell surface receptor molecules for RVF virus."

2. Application of the ABC Complex Immuno-cytochemical Procedure to Paraffin Sections of Ticks Infected with RVF Virus

To date our attempts to apply the ABC procedure to detect RVF viral antigen in paraffin sections of ticks have not worked.

3. Application of Immunogold Techniques for Use in Ultrastructural Studies of Mosquito Cells Infected with RVF Virus

Results of application of a post-embedding immunogold-protein A technique for locating RVF viral antigen at the ultrastructural level have been equivocal.

IV. Studies in Progress

A. Evaluation of the effects of several possible environmental/ physiological parameters which may influence infection and dissemination of RVF virus in Culex pipiens. (Among these factors will be dose, temperature, interrupted feeding followed by a second blood meal, sugar-feeding history, availability of water, blood meal size, number of gonotropic cycles preceding infectious blood meal, virus strain, age of adult, etc.)

B. Evaluation of the significance of the intussuscepted foregut (IF) route of dissemination from the gut lumen.(We plan to carry out an experiment in which the same viral dose will be provided orally via pledget and rectally via enema. If our hypothesis that the IF is a major route of egress of virus from the gut is true, we would expect, on the average, earlier and more extensive dissemination of virus in specimens which received virus via the oral route.)

C. Immunocytochemical study of the effects of blood-feeding on infection of the midgut from the hemocoel of IT infected Culex pipiens and on the dynamics of infection of the midgut from the hemocoel. (This is also pertinent to our hypothesis that the basal lamina acts as a midgut escape barrier.)

D. Immunocytochemical study of the salivary glands of IT infected Anopheles stephensi [work at USAMRIID has indicated the probable operation of a "salivary gland barrier" in this species (M. Turell, personal communication)]

E. Continuation of attempts to adapt the ABC complex immunocytochemical technique in studies of RVF virus in ticks.

F. Application of cDNA probes for in situ localization of RVF virus in mosquito sections. (Biotinylated DNA probes complementary to segments of the RVF virus genome have been developed by Dr. F. Knauert and Dr. M. T. Vahey at USAMRIID. Dr. Knauert's probe has been applied successfully in the in situ detection of RVF virus genome in mouse liver sections. We are currently attempting to adapt his protocol for use with serial paraffin sections of mosquitoes.)

G. Development of the application of immunogold techniques to detect RVF viral antigen and virions in situ in Culex pipiens and other mosquito species. (We are studying RVF morphogenesis in various tissues in Culex pipiens especially the midgut, intussuscepted foregut, fat body, ganglia and salivary glands. The cells of the

intussuscepted foregut will be especially useful in detailed morphogenesis studies since the frequency of infection in intrathoracically inoculated specimens approaches 100%. We also plan to examine the tracheael system and skeletal muscle for the presence of virus since the results of immunocytochemical study have been equivocal.)

H. Histological & Ultrastructural studies. (We are continuing to study the histology and ultrastructure of the mosquito proventriculus, primarily due to our hypothesis that virus can exit the midgut lumen via cells within this structure.)

I. Identification and characterization of mosquito cell surface receptors for RVF virus.

J. Development of monograph on mosquito histology and ultrastructure. (Most of our immunocytochemical studies and ultrastructural studies should generate valuable photographs and structural insights that will be useful in the production of this monograph.)

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