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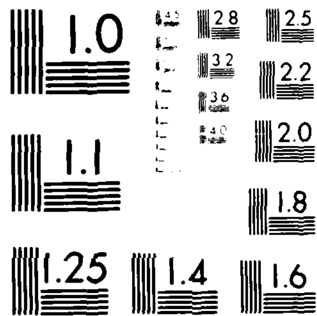

MICROCOPY RESOLUTION TEST CHART

AD-A149 459

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Report No. 44-95-0182-TSI004

DEVELOPMENT OF SPECIAL BIOLOGICAL PRODUCTS (U)

Annual Progress Report

by

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January 1982

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-78-C-8018

The Salk Institute
Government Services Division
P.O. Box 250
Swiftwater, PA 18370

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Rift Valley Fever
Tissue Culture
FRhL-2
MRC-5
Venezuelan Equine Encephalomyelitis (VEE)

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

A. Tissue Culture

Two lots of FRhL-2 and one of MRC-5 were stabilized and frozen. A new production seed for MRC-5 was processed at Pass 14. Two production seeds (P11 and P12) and one production lot of *A. albopictus* were prepared. Five cell lines for use in testing were added to our inventory. Four lots of MRC-5 plus a partial lot were depleted by shipment to USAMRIID. A total of 3409 amps of frozen cells were processed.

19. Continued

Storage Stability
Chikungunya
HA Antigen
Eastern Equine Encephalitis (EEE)
Western Equine Encephalitis (WEE)
Dengue
Q Fever
Tularemia Vaccine
Korean Hemorrhagic Fever (KHF)

20. Continued

B. VEE (C-84) Vaccine Development

A total of 93,370 doses of Lot C-84-7 was prepared making the total number of doses produced 568,310.

C. VEE Vaccine Potency Testing

Potency testing was completed on 17 different lots and/or runs of VEE vaccine.

D. WEE Vaccine Development

Three WEE virus isolates were evaluated for vaccine antigens. One lot of antiserum was made in rabbits for use in certification of the viral seed.

E. WEE Vaccine Potency Testing

Lot 1-81 (TSI-bulk-liquid) and Lot 2-74 (USAMRIID-final product) were potency tested.

F. Rift Valley Fever Vaccine (WRAIR) Potency Testing

Lots 18 and 19 prepared during June 1964 at WRAIR were potency tested.

G. Rift Valley Fever (RVF) Vaccine Prepared at The Agouza Institute

RVF vaccine, Lots 52, 53, 60 and 63 prepared by The Agouza Institute was tested.

H. Q Fever Vaccine Stability Testing

Lots 2 and 4 were potency tested after storage at -20°C for 9 years.

I. Attenuated Chikungunya Vaccine Safety Test in Rabbits and Mice

Safety testing was performed on attenuated Chikungunya virus received from USAMRIID.

J. Tularemia Vaccine Stability Studies

Lots 2, 4 and 9 stored at -20°C for at least 17 years were potency tested.

K. RVF Vaccine Development

Three strains of RVF were compared for growth in five cells available in this laboratory. The ZZ501 RVF virus was passed in certified FRhL-2 cells for a production seed and testing is in progress to certify the seed.

L. RVF Vaccine Potency Testing

A modified potency test was performed in accordance with instructions received from USAMRIID.

M. Rift Valley Fever Antisera

Rabbit antisera were produced against inactivated Zagazig and H1849 strains. Rabbit antisera were also prepared using live. Entebbe strain as the immunogen.

N. HA Antigens

One lot of EEE, Lot 1-81, was prepared and dried and one lot of WEE HA antigen (Lot 1-81) was dried. A total of 504 amps of RVF HA antigen (Lot 2-80) was shipped to Dr. Shope.

O. Dengue Antiserum

One lot of Dengue antiserum was prepared.

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20. ABSTRACT (Continued)

P. Drug Screening Program

Mouse protection tests were performed against RVF (Entebbe strain) virus with 13 of 15 drugs received. One MPT was conducted against VEE (Trinidad strain) virus using Ly 122771. Plaque reduction tests were completed using RVF virus and 14 drugs. The same test was used to test 21 drugs against VEE virus and 13 drugs against Pichinde virus. One yield inhibition test was performed against RVF virus.

Q. Korean Hemorrhagic Fever

A KHF laboratory was established this year. Production of diagnostic reagents began and continues. One lot of rabbit antiserum was prepared.

R. Immunization Program

Seven new employees were immunized.

Unclassified
Security Classification

Summary

A. Tissue Culture

Two production lots of FRhL-2 and one of MRC-5 were stabilized and frozen this year. A new production seed for MRC-5 was processed at Pass 14. Two production seeds (i.e. P11 and P12) and one production lot of *Aedes albopictus* were prepared. Five cell lines for use in testing (i.e. Vero Clone 008, CER4, PK15, Bovine turbinate and S-549) were added to our inventory during this period. Four lots of MRC-5 plus a partial lot were depleted by shipment to USAMRIID. The PPLO laboratory was moved to larger quarters in Suite 3 to handle the increased level of work. A total of 3409 amps of frozen cells were processed.

B. VEE (C-84) Vaccine Development

One lot of vaccine (C-84-7) was prepared this year to satisfy the volume requirement. A total of 93,370 doses (0.5 ml) of this lot were dried and placed at -20°C, making the total number of doses 568,310. Of the 2,671 rolling cultures processed from 13,576 embryonated eggs, none were lost due to contamination and a total of 10 were discarded due to cracked flasks or defective containers.

C. VEE Vaccine Potency Testing

Lots C-84-1, C-84-1A (MNLBR 109), C-84-2 Run 1 and C-84-3 Run 1 (TSI-GSD 205) of VEE vaccine were potency tested in guinea pigs by HI and protection and challenge. This was followed by Lots C-84-2 Runs 1,2,3, C-84-4 Runs 1,2,3, C-84-4 Runs 1,2,3, C-84-5 Runs 1,2,3,4 (TSI-GSD 205). Potency testing of Lots C-84-6 Runs 1,2,3,4 and C-84-7 Runs 1,2,3 is in progress. A comparison of the challenge virus titrated in guinea pigs vs the mouse gave identical LD_{50} titers.

D. WEE Vaccine Development

Three WEE virus isolates were evaluated for vaccine antigens. Tissue culture passages in primary CEC of these viruses did not produce potent vaccines, however, one of the viruses produced potent inactivated vaccine following one passage in chicken embryos. The egg passage seed virus was used to produce a 25-liter lot of vaccine. This vaccine has been shown to be a safe, potent, inactivated preparation. The seed virus is in the process of being certified. One lot of WEE antiserum was made in rabbits for use in certification of the viral seed.

E. WEE Vaccine Potency Testing

Lot 1-81 (TSI-bulk-liquid) and Lot 2-74 (USAMRIID-final product) of WEE vaccine were potency tested in guinea pigs by HI and protection followed by challenge. Runs 1 and 2 of Lot 1-81 (TSI-GSD 210) are currently on potency test.

F. Rift Valley Fever Vaccine (WRAIR) Potency Testing

Lots 18 and 19 of Rift Valley Fever Vaccine prepared during June 1964 at WRAIR were potency tested in accordance with instructions contained in the 26 March 1981 letter from WRAIR.

G. Rift Valley Fever (RVF) Vaccine Prepared at The Agouza Institute

Rift Valley Fever Vaccine, Lots 52, 53, 60 and 63, prepared by The Agouza Institute, Cairo, Egypt was tested and a report was submitted to USAMRIID.

H. Q Fever Vaccine Stability Testing

Lots 2 and 4 of Q Fever Vaccine (NDBR 105) were potency tested after storage at -20°C for nine years.

I. Attenuated Chikungunya Vaccine Safety Test in Rabbits and Mice

In accordance with instructions received from USAMRIID safety tests on attenuated Chikungunya virus were performed in rabbits and mice.

J. Tularemia Vaccine Stability Studies

Tularemia Vaccine (NDBR 101) Lots 2, 4 and 9 stored at -20°C for at least 17 years were potency tested.

K. RVF Vaccine Development

Three strains of RVF were compared for growth in five cells available in this laboratory. Each of the viruses yielded similar titers in all cell lines, however the Entebbe virus harvest had the lowest titer and the ZZ501 virus the highest, the SA-75 was intermediate. The ZZ501 RVF virus was passed in certified FRhL-2 cells for a production seed and testing is in progress to certify the seed for vaccine preparation.

L. RVF Vaccine Potency Testing

The potency test described in this report was performed in accordance with instructions received from USAMRIID and differs from RVF vaccine potency tests previously conducted at TSI in the following respects; the Zagazig 501 strain replaced the Entebbe strain as challenge virus, 42-day-old mice were used, and one dose of vaccine was administered instead of two doses.

M. Rift Valley Fever Antisera

Rabbit antisera were produced against inactivated Zagazig and H1849 strains of RVF virus. Rabbit antisera (TSI-GSD 201 Lot 3) were also prepared using live, Entebbe strain, RVF virus as the immunogen.

N. HA Antigens

One lot of EEE, Lot 1-81, HA antigen was prepared and dried and one lot of WEE HA antigen (Lot 1-81) was dried. Safety testing in suckling mice remains to be done. A total of 504 amps of RVF HA antigen (Lot 2-80) was shipped to Dr. Shope.

O. Dengue Antiserum

Dengue, type 1 virus, Hawaii strain, received from Dr. Shope at the Yale Arbovirus Research Unit was passed in suckling mice. The infected mouse brain material served as a live immunizing agent that was then injected into rabbits. Sera obtained from inoculated rabbits passed our testing procedures and were dispensed.

P. Drug Screening Program

Virus stocks were prepared and assays established for the four viruses to be used in drug screening. Mouse protection tests (MPT) were performed against RVF (Entebbe strain) virus with 13 of 15 drugs received from USAMRIID. One MPT was conducted against VEE (Trinidad strain) virus using Lv 122771. Plaque reduction tests (PRT) were completed using RVF virus and 14 drugs. The same test was used to test 21 drugs against VEE virus and 13 drugs against Pichinde virus. One yield inhibition test (YIT) was performed using Atromid-S against RVF virus. The OCT-541 strain of JBE virus was standardized.

Q. Korean Hemorrhagic Fever

A KHF laboratory was established this year in Room E Suite III with the installation and final testing of a Class II Type A Nu-Aire Laminar Flow Biological Safety Cabinet. Production of diagnostic reagents in support of the KHF program began and continues. One lot of rabbit antiserum was prepared.

R. Immunization Program

Seven new employees were immunized with Q Fever, RVF, EEE, VEE and WEE vaccines and six received Tularemia Vaccine.

Foreword

The authorization for the work contained herein was authorized under Contract No. DAMD17-78-C-8018, titled, "Development of Special Biological Products".

This annual report covers the period of January 1, 1981 through December 31, 1981. In conducting the research described in this report, the investigators adhered to the "Guide for the 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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Tissue Culture

I. Introduction

Two production lots of FRhL-2 and one of MRC-5 were stabilized and frozen this year. A new production seed for MRC-5 was processed at Pass 14. Two production seeds (i.e. P11 and P12) and one production lot of *Aedes albopictus* were prepared. Five cell lines for use in testing (i.e. Vero Clone 008, CER4, PK15, Bovine turbinate and S-549) were added to our inventory during this period. Four lots of MRC-5 plus a partial lot were depleted by shipment to USAMRIID. The PPLO laboratory was moved to larger quarters in Suite 3 to handle the increased level of work. A total of 3409 amps of frozen cells were processed.

II. Process Studies

A. Production Cells

1. FRhL-2 Diploid Cell Line

Two production lots of FRhL-2, Lots 26 and 27, were prepared and certified as summarized in Table 1. The chromosome analyses are shown in Table 2.

A total of 80 amps of FRhL-2 was used for viral seed preparations and testing procedures.

Investigation into a possible contamination of Lots 19 and 20 FRhL-2 was made by recovering one ampule of each from the historical file. Fluids from these cultures passed in primary duck or LLC-MK2 cells, subpassed into embryonated eggs or suckling mice and then subpassed again on LLC-MK2 cells has not implicated either lot as having a contaminant.

2. Primary Chick Embryo Cells

A total of 4[illegible]6 rolling cultures were prepared for a sixth lot of C-84 VEE vaccine. Several small batches of CEC were prepared for testing use.

3. MRC-5 Diploid Cell Line

All testing was completed on Lot 12 cells as shown in Table 3. The chromosome analysis is given in Table 4. A new production seed (PS2) was prepared and 124 ampules containing 11 x 10^6 cells at 90 percent viability were frozen at P14. This seed was used to initiate a new production lot to be harvested at P13.

One uncertified lot (#14) was processed and 450 amps were shipped to USAMRIID for experimental use. Processing and limited testing data on this lot are summarized in Table 5. In addition to the above, 659 ampules of various lots were shipped to USAMRIID, depleting Lots 5, 6 and 7 and part of Lot 9.

4. Aedes albopictus (C6/36)

Two production seeds (PS) were prepared from different ampules of P8 cells obtained from WRAIR. The second PS was prepared after a positive PPLO test was reported. This later was found to be a false-positive due to the horse serum being used at the time.

One production lot of *Aedes albopictus* was prepared from PS1 at Pass 15. Testing is in progress.

Production lot 2 was initiated from PS2, to be harvested at Pass 14 by using additional ampules of PS2 for the first passage. This work is progressing. Karyological work, to supplement the initial study (P11-March 21, 1980-100 counts/20 idiograms-letter to Dr. F. Cole) is currently in progress on Passages 14, 16 and 17.

Shipment of 10 amps of PS1 was made to WRAIR for testing and 25 amps were used in testing and production.

5. Primary Duck Cells

One lot of primary duck cells (PD2) was prepared from Spafas duck eggs (13 day). A total of 339 amps, each containing 145×10^6 cells at 72 percent viability was produced. The cells passed tissue culture safety, bulk sterility, TB and embryonated egg safety tests. However, the cells did not sheet well and did not maintain well. We believe the use of Reheis enzyme at 0.025 percent was too strong and depleted the harvested cells of vitality. This lot will be replaced.

B. Test Cells

1. Vero Clone 008

This line was processed into 444 ampules at P26. Each amp contains 10×10^6 cells at 93 percent viability. Sixty amps were sent to USAMRIID.

2. CER4

CER4 cells were frozen at pass 17 (78 amps) for use in an RVF growth curve experiment. Eight amps have been used.

3. PK15 (CCL 33)

This cell was passed and frozen (104 amps) at P136 for use

in BVD virus testing. The frozen amps were checked by Dr. Van Deusen at Ames and found to be adventitious-agent free.

4. <u>Bovine Turbinate Cells</u> (CRL1390)

A small harvest of these cells was made at P24 (104 amps). These cells, grown on horse serum are for use in BVD virus tests. A summary of preparation is given as Table 6 .

5. <u>A549 (CCL 185)</u>

A549 was processed and frozen at P81 for use in KHF virus work. Preparation is shown in Table 7 .

6. <u>Miscellaneous</u>

Small numbers of amps of KB, LLC-MK2, RK-13, Vero and BHK21 were used for safety test and assay purposes. These cells are usually maintained in continuous culture or are restarted from monolayers frozen in-situ.

A new lot of serum (#100319) which was certified BVD-free by Dr. McClurkin of Ames was obtained from Sterile Systems to replace, bottle-for-bottle, Lot #100277 which was contaminated with BVD.

One fermentor ($3\frac{1}{2}$ liters) Vero cells grown on Cytospheres was infected with EEE virus but the yield of virus was low. Some of the cells on the spheres became detached during rinsing, prior to infecting. This may have contributed to the result.

C. <u>Equipment/Laboratory</u>

1. <u>Cozzoli Ampule Filler-Sealer</u>

The Cozzoli ampule filler-sealer was used for three cell production runs. The efficiency has increased to 95-98 percent good seals due to experienced handling. Remaining to be added to the system is a device (either magnetic or physical movement) to maintain the cells in homogeneous suspension during filling.

2. The FFL[illegible] laboratory was moved from Suite 4, small laboratory to Suite [illegible], Room A, doubling the size. As soon as a laminar flow hood, incubator, refrigerator-freezer, water bath and microscope are obtained, work on fluorescence-detection of <u>Mycoplasma</u> can be instituted on a routine basis for screening of materials and cells.

III. Cell Inventory and Use-1981

The inventory is given as Table 8 , showing our current stocks. A total of 1179 ampoules were shipped and 214 were used here for testing and seed preparations. A total of 2530 amps for vaccine work and 879 amps for testing were produced during the year.

IV. Conclusion

We have increased the efficiency of testing somewhat this year with personnel gaining more experience. A few problems with mold contamination occurred which, in all probability, was caused by a contaminated water bath. We are currently using Wescodyne in the baths to aid in this problem.

Liquid nitrogen freezer space will become a problem in the coming year as additional cell types come on stream and inventories are built to a reasonable level.

Problems with SPAFAS duck embryos in producing good, viable cells remain. We will concentrate on this problem in the near future, as well as developing a standard method for preserving primary CEC, as time allows.

Table 1

Preparation and Testing of FRhL-2 P16

Item	Result Lot 26	Result Lot 27
No. bottles harvested	719	959
Surface area (cm^2)	107,850	143,850
Total cells ($\times 10^{10}$)	1.4	1.2
Cells/cm^2 ($\times 10^5$)	1.3	0.85
No. amps	535	768
Cells/amp ($\times 10^6$)	26.8	16.04
Viability (%)	93	91
Sheetability -- 1 amp - 10 x 75 cm^2	3 days	3 days
1 amp - 10 x 150 cm^2	4 days	4 days
1 amp - 850 cm^2 roller	4-5 days	4-5 days
Bulk sterility	Sterile	Sterile
2 week-hold of 1-2% cell sample after harvest	Normal	Normal
30-day hold of harvest fluids	Sterile	Sterile
PPLO	Negative	Negative
Hemadsorption: cells from sheetability	Negative	Negative
2 week-hold-harvest cells	Negative	Negative
M. tuberculosis (Lowenstein-Jensen)	Negative	Negative
Tissue culture safety -- a) RK-13	Passes	Passes
b) CV-1 & subpass	Passes	Passes
c) MRC-5	Passes	Passes
d) CEC	Passes	Passes
e) LLC-MK2 & subpass	Passes	Passes
Egg safety (allantoic)	Passes	Passes
Tumorigenicity (newborn hamster/ALS)	Passes	Passes
Karyology	Normal Diploid	Normal Diploid

Table 2

Chromosome Analysis on Two Lots of FRhL-2 (P17)

No. of chromosomes	Lot 26	Lot 27
	(no. of cells)	
40	5	3
41	8	5
42	86	92
43	1	
Polyploidy	1.6%	1.0%
Aberration	6%	7%
Total No. Cells Counted	100	100
Modal Chromosome No.	42	42

Table 3

Preparation and Testing of MRC-5 P23 Lot 12

Item	Result
No. bottles harvested	952
Surface area (cm^2)	142,800
Total cells ($x\ 10^{10}$)	1.6
Cells/cm^2 ($x\ 10^5$)	1.1
No. amps (automatic sealer-15% reject)	765
Cells/amp ($x\ 10^6$)	20.6
Viability (%)	92
Sheetability -- 1 amp - 10 x 75 cm^2	3 days
1 amp - 850 cm^2 roller	3 days
1 amp - 2 x 850 cm^2 rollers	5 days
Bulk sterility	Sterile
2 week-hold of 1-2% cell sample after harvest	Normal
30-day hold of harvest fluids	Sterile
PPLO	Negative
Hemadsorption: cells from sheetability	Negative
2 week-hold-harvest cells	Negative
M. tuberculosis (Lowenstein-Jensen)	Negative
Tissue culture safety--a) RK-13	Passes
b) CV-1 + subpass	Passes
c) FRhL-2	Passes
d) CEC	Passes
Egg safety (allantoic)	Passes
Tumorigenicity (newborn hamster/ALS)	Passes
Karyology	Normal diploid

Table 4

Chromosome Analysis on MRC-5 Lot 12 (P24)

No. of Chromosomes	No. of Cells
45	7
46	93
Polyploidy	2.8%
Aberration	6%
Total No. Cells Counted	100
Modal Chromosome No.	46

Table 5

Preparation of an Uncertified Lot of MRC-5 (Lot 14)

Day	Passage	No. of 150 cm^2 Flasks	Surface Area (cm^2)	Condition
0	18	3	450	Antibiotic-free
5	19	9	1,350	Antibiotic-free
8	20	27	4,050	17 cultures antibiotic-free
12	21	81	12,150	Antibiotics present
15	22	243	36,450	" "
18	Media change	243	36,450	" "
21	23	729	109,350	" "
26	Harvest	719*	107,850	" "

Harvest: Total cells 1.39×10^{10}
Cells/cm^2 1.3×10^5

Freezing: 463 amps at 30×10^6, 96% viable (heat-sealable-plastic)

Testing: Bulk sterility: sterile
*2 week hold-10 cultures: normal morphology
Hemadsorption: negative
Sheeting: 1 amp sheets:

20 x 75 cm^2	3 days	20 x 150 cm^2	4 days
10 x 75 cm^2	2 days	10 x 150 cm^2	3 days
5 x 75 cm^2	1 day	5 x 150 cm^2	2 days

PPLO: Negative
TB: Negative

Table 6

Preparation of Bovine Turbinate (CRL 1390)* Cells for Use in Testing

Passage	Surface Area (cm^2)	Day	Comment
19	75	0	MC on days 1,2,6,11,14
20	150	20	MC on day 26
21	300	28	
22	900	36	
23	2700	43	
24	8100	50	
	7800	57	Harvest

No. ampules: 104

Cells/amp/viability: 6.1×10^6/96%

Cells/cm^2 0.81×10^5

*Grown on horse serum.

Table 7

Preparation of A549 (CCL 185) Cells for Use in Testing

Passage	Surface Area (cm^2)	Day	Comment
78	75	0	
79	375	7	
80	2250	11	
81	6750	15	
		18	Harvest

No. amps: 148

Cells/amp/viability: 8.3×10^6 97%

Table 8

Cell Inventory and Use - 1981

Item No.	Cell	Lot No.*	Pass.	Date Frozen	Ampoule Cell Count (x 10⁶)	Viability (%)	No. Amps Jan 80	Amps Shipped	Amps Used	Current Inventory	Use
1	FRhL-2	[illegible]	[illegible]	[illegible]/14/7[illegible]	[illegible]	94-98	82		3	79	Certified Cells for Vaccine Preparation
		4-OPS	1[illegible]	11/2[illegible]/76	8.0	75	284			284	
		16	17	2/2[illegible]/78	2[illegible].0	96-98	288			288	
		21**	1[illegible]	3/20/7[illegible]	2[illegible].8	93	297			297	
		24	1[illegible]	4/2/8[illegible]	31.4	90	449			449	
		26	16	2/10/81	2[illegible].8	93	---		37	498	
		27	16	2/24/81	16.0	91			40	728	
2	FCL-7	Seed	"	12/22/77(Rec'd)	--	--	1			1	
		OPS	16	5/17/77	5.4	90-94	193			193	
3	IMR-90	M[illegible]	10	5/16/77	4.9	94	180			180	
		P[illegible]	14	6/1/77	3.4	99	46		1	45	
		1	21	11/14/77	37.7	94-97	305		4	301	
4	IMR-91	MS	1[illegible]	8/31/78	5.2	100	98			98	
5	MRC-5	P[illegible]-1	17	6/7/77	7.0	100	24		24	0	
		P[illegible]-2	14	12/28/81	11.0	90			4	120	
		5	23	1/11/78	42.0	92-96	244	244		0	
		6**	23	8/6/7[illegible]	33.0	93	12	12		0	
		7**	23	[illegible]/14/7[illegible]	[illegible].5	96	72	72		0	
		[illegible]	23	6/1[illegible]/80	21.3	98	529	331	5	193	
		1[illegible]	23	7/1[illegible]/80	23.6	98	379		3	376	
		1[illegible]	23	11/4/8[illegible]	29.6	92	621		14	607	
		14**	23	6/23/81	30.0	96		450		0	
6	PLC (Duck)	1	Primary	2/26/75	152.0	93	6		2	4	
7	DK (Dog Kidney)	Pow	Primary	4/5/77(Rec'd)			1375			1375	
8	Aedes albo-pictus (C6/36)	PS1	11	7/27/81	62.	76		10	1	93	
		PS2	1[illegible]	12/17/81	11.2	65			16	93	
		1	15	12/15/81	13.6	71			8	436	

Table [illegible]

Cell Inventory and Use - 1981

Item No.	Cell	Lot No.*	Pass	Date Frozen	Ampoule Cell Count (x 10^6)	Viability (%)	No. Amps Jan 80	Amps Shipped	Amps Used	Current Inventory	Use
9	[illegible]-1	--	76	[illegible]/75	14.0	84-87	19			19	Test Cells
10	[illegible]V-1	--	[illegible]	12/21/76	1.3	85	10			10	
			36	10/20/78	--		67			67	
11	[illegible]B	--	--	3/18/75	14	89-98	48		3	45	
12	[illegible]	--	[illegible]	2/11/75	4.0	78	32		1	31	
13	[illegible]	--	73	6/16/75	4.0	83	37		2	35	
14	Vero	--	122	4/28/[illegible]	2.0	82	41		7	34	
		--	13[illegible]	8/30/79	32.4	96	81			81	
		[illegible]-1	[illegible]	11/12/81	10.0	93		60	14	370	
15	[illegible]	--	57	5/14/79(Rec'd)	--	--	5			5	
		--	58	6/12/79	30.0	98	21		3	18	
16	[illegible]	--	[illegible]	12/18/79(Rec'd)			1			1	
			[illegible]	1/10/80	5.1	95	87			87	
17	[illegible]P-4	1	17	3/30/81	25.7	99			8	71	
18	[illegible]-15	1	13[illegible]	6/24/81	25.6	97			3	101	
19	Bovine turbinate	1	24	7/31/81	5.2	96				104	
20	A-549	1	81	10/2/81	8.3	97			9	140	

*PS = Production Seed; OPS = Old Production Seed; MS = Master Seed
**Shipped as uncertified cells.

VEE (C-84) Vaccine Development

I. Introduction

One lot of vaccine (C-84-[illegible]) was prepared this year to satisfy the volume requirement. A total of [illegible],370 doses (0.5 ml) of this lot were dried and placed at -20°C, making the total number of doses 568,31[illegible]. Of the 2,671 rolling cultures processed from 13,576 embryonated eggs, none were lost due to contamination and a total of 10 were discarded due to cracked flasks or defective containers.

II. Vaccine Production

A. A sixth lot of VEE C-84 was produced this year to complete the volume requirement. Production data and testing are shown in Tables 9 and 10 along with the current status of the other five lots. Potency testing remains to be completed.

B. The vaccine preparation method worked well throughout the preparation of this vaccine. Sterility precautions instituted throughout the procedures were adequate with no contamination being detected in preparing the 2,671 rolling cultures from 13,576 embryonated eggs.

Table [illegible]

Production and Safety Tests on Six Lots of C-84 VEE Vaccine
(Bulk Fluid Virus and Vaccine Fluids)

Procedure[1]	C-84-2	C-84-3	C-84-4	C-84-5	C-84-6	C-84-7
No. embryos	1[illegible]	2129	2091	2332	2555	2510
No. cultures: total[2]	404	410	423	476	482	476
infected	377	383	400	457	466	437
Vol. virus fluid (L)	55.0	59.8	54.9	70.0	64.8	61.4
Virus titer[3]	10.6/9.0/9.8	10.1/9.4/10.5	9.7/8.9/9.7	9.5/9.5/9.9	10.3/9.9/9.3	9.7/9.[illegible]/10.0
Monitor test:						
0-time	9.4/8.4/9.8	8.9/7.6/9.7	9.0/8.0/8.6	9.0/9.1/9.2	8.7/7.9/<7.0	8.5/7.9/9.6
6-hr.	1.1/2.8/<1.0	1.4/2.1/<1.0	2.5/2.9/2.7	4.5/3.3/1.9	3.5/2.1/<1	<2.9/<1.0/2.2
24-hr.	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U
72-hr.	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U	<Und/<U/<U
Residual formalin (%)	0.09	0.114	0.096	0.116	0.100	0.098

[1] The following tests were done on each lot and the results satisfactorily met or exceeded established standards of CFR 21 [illegible]: In process sterility (bulk virus and control fluid); PPLO, TB (cultural); HAd; residual live virus.

[2] Average volume of virus fluid/roller bottle (850 cm^2) = 145 ml.

[3] Titer: Log_{10}/0.5 ml. CPE, TC, CPE/Plaque, CEC/Suckling Mice, IC.

Table 10

Final Container Tests on Six Lots of C-84 VEE Vaccine, Inactivated, Dried

Lot Number/	Run Number	Residual Formalin (%)	pH	Data[1] Moisture (%)	Potency[2] (ED_{50}, ml)	No. Vials Dried[3]
C-84-2	1	0.005	6.85	0.26	0.02	1927
	2	0.006	6.90	0.35	0.008	820
	3	0.006	6.90	0.28	0.017	467
C-84-3	1	0.005	6.70	0.25	0.006	1732
	2	0.006	6.73	0.20	0.007	978
	3	0.005	6.68	0.18	0.009	987
C-84-4	1	0.005	6.34	0.24	0.044[4]	1875
	2	0.005	6.72	0.23	0.044	932
	3	0.006	6.70	0.21	0.031	925
C-84-5	1	0.015	6.90	0.29	0.011	1834
	2	0.014	7.14	0.23	0.010	982
	3	0.006	7.03	0.22	0.011	683
	4	0.008	7.13	0.20	0.013	479
C-84-6	1	0.003	6.65	0.26	Not Done	1838
	2	0.003	6.75	0.27	"	729
	3	0.003	6.90	0.98	"	795
	4	0.003	7.11	0.57	"	820
C-84-7	1	0.005	6.88	0.38	Not Done	1877
	2	0.005	7.05	0.18	"	948
	3	0.005	6.94	0.22	"	942

[1] The following tests were done on each run and the results satisfactorily met, or exceeded established standards of CFR 21: bulk and final container sterility; general safety.

[2] Potency value determined by probit analysis on survivors in an antigen-extinction test in guinea pigs (2 doses IM, 0.5 ml) with Trinidad challenge.

[3] Run 1 Dried-5.5 ml/vial
Runs 2 and 3 (4) dried - 21 ml/vial

[4] Repeat potency tests on Run 1 gave ED_{50} values of 0.013 and 0.011.

VEE Vaccine Potency Testing

I. Introduction

Lots C-84-1, C-84-1A (MNLBR 109), C-84-2 Run 1 and C-84-3 Run 1 (TSI-GSD 205) of VEE vaccine were potency tested in guinea pigs by HI and protection and challenge.

This was followed by potency testing of Lots C-84-2 Runs 1,2,3 C-84-3 Runs 1,2,3, C-84-4 Runs 1,2,3, C-84-5 Runs 1,2,3,4 (TSI-GSD 205) Vee vaccine in guinea pigs by HI and protection and challenge.

Potency testing of Lots C-84-6 Runs 1,2,3,4 and C-84-7 Runs 1,2,3 is in progress.

A comparison of the challenge virus titrated in guinea pig vs the mouse gave identical LD_{50} titers.

II. Potency Test Results

Potency testing was performed on four (4) lots of VEE vaccine. The results in Table 11 show that by both HI testing and after challenge with the Trinidad strain of VEE Lot C-84-1 was the superior vaccine since all six animals survived challenge and 50% had significant antibody rises following immunization with a 1:25 dilution of vaccine. Both groups of animals given a 1:25 dilution of Lot C-84-1A and Lot C-84-3 Run 1 vaccines had the same number of survivors (5/6) after challenge. However, the latter vaccine was more effective in antibody production since it converted 33% of the animals given a 1:25 dilution of vaccine while in the former group none was converted. Lot C-84-2 Run 1 had the least potency by survival rates (3/6) in the group given vaccine diluted 1:25 and a 17% and 0% conversion in animals given vaccine diluted 1:5 and 1:25, respectively.

Four (4) lots of VEE vaccine consisting of a total of 13 runs, were also potency tested. The results in Table 12 show by all criteria given, that the 3 runs of VEE lot C-84-3 were superior, followed by the 4 runs of Lot C-84-5 and the 3 runs of C-84-2. The poorest vaccine noted was C-84-4 with all 3 runs giving the poorest results. Great discrepancies were noted in comparing the number of surviving guinea pigs given various vaccines diluted 25 times followed by challenge. Lot C-84-4 had 10%-20% survivors while the other 3 vaccines usually had from 80-100% survivals. The percent serological conversions, and amount of vaccine required to protect guinea pigs from death by probit analysis and Reed & Muench also indicated Lot C-84-4 to be inferior to the other vaccines tested.

Potency testing of Lots C-84-6 Runs 1,2,3,4 and C-84-7 Runs 1,2,3 is in progress.

Table 11

VEE Vaccine Potency Testing

VEE Vaccine	Survivors/Total Immunized with Vaccine Dilutions at		Serological Conversions by HI from Guinea Pigs Given Vaccines Diluted	
	1:5	1:25	1:5	1:25
Lot C-84-1	6/6	6/6	67%	50%
Lot C-84-1A	6/6	5/6	67%	0%
Lot C-84-2 Run 1	6/6	3/6	17%	0%
Lot C-84-3 Run 1	6/6	5/6	67%	33%

Table 12

VEE Vaccine Potency Testing

VEE Vaccine	Survivors/Total Immunized with Vaccine Dilutions at		Serological Conversions* by HI from Sera of Guinea Pigs with Vaccine Dilutions at		ED_{50}(ml)	
	1:5	1:25	1:5	1:25	Probit	R&M
Lot C-84-2 Run 1	9/9	5/10	78%	0%	0.020	0.019
Run 2	10/10	9/10	50%	0%	0.012	0.013
Run 3	10/10	8/10	10%	0%	0.018	0.015
Lot C-84-3 Run 1	10/10	10/10	80%	40%	0.005	0.006
Run 2	10/10	9/9	100%	40%	0.006	0.006
Run 3	10/10	9/10	100%	10%	0.007	0.008
Lot C-84-4 Run 1	9/10	1/9	10%	10%	0.044	0.045
Run 2	9/10	1/10	10%	0%	0.044	0.045
Run 3	10/10	2/9	0%	0%	0.029	0.036
Lot C-84-5 Run 1	10/10	9/10	50%	20%	0.011	0.010
Run 2	10/10	10/10	90%	10%	0.008	0.008
Run 3	10/10	10/10	67%	10%	0.011	0.013
Run 4	10/10	10/10	70%	10%	0.011	0.014

*Conversion $\geq$1:20

WEE Vaccine Development

I. Introduction

Three WEE virus isolates were evaluated for vaccine antigens. Tissue culture passages in primary CEC of these viruses did not produce potent vaccines, however, one of the viruses produced potent inactivated vaccine following one passage in chicken embryos.

The egg passage seed virus was used to produce a 25-liter lot of vaccine. This vaccine has been shown to be a safe, potent, inactivated preparation. The seed virus is in the process of being certified.

One lot of WEE antiserum was made in rabbits for use in certification of the viral seed.

II. Comparison of Three WEE Viruses for Vaccine Seeds

Three WEE viruses isolated at Fort Collins, Colorado, from mosquitos were received from USAMRIID as first passage tissue culture fluid (certified chick embryo cells). A second passage in certified CEC was done at The Salk Institute-GSD and the terminal dilutions were stored for seed in further passages.

The third and fourth passages were made in CEC using a 10^{-3} dilution of the preceding passage. Inactivated vaccines prepared at the fourth passage were impotent in the mouse protection potency test as shown in Table 13 .

The terminal dilution of virus CM4884. 10^{-7}, was used to inoculate 8-day chick embryos (SPAFAS) by the yolk sac route. The $10^{-[illegible]}$ dilution of this virus fluid killed seven of nine embryos in 24 hours and the [illegible] percent embryo suspension prepared from the embryos had a titer of 10.7[illegible] log_{10} in CEC. The data as shown indicate that the egg passage increased the titer 100-fold over the CEC third passage. An inactivated vaccine prepared in CEC at the fourth passage with the embryo suspension was potent in the mouse protection test.

III. WEE Vaccine, Lot 1-81

The chick embryo suspension virus CM4884 (P3) was used to seed roller bottles (850 cm^2) at a dilution of 10^{-5} log_{10} (MOI = 0.0015). Twenty-five liters of virus fluid (M-199-2% HSA) was harvested from 18[illegible] [illegible] cultures at 24 hours post infection. The virus fluid was inactivated with 0.05 percent

formalin. Tests for residual live virus in suckling mice on the seven-day fluid showed the presence of live virus. The formalin concentration was increased to 0.1 percent with additional incubation at 37°C. The retests for live virus were negative after this procedure and the vaccine was freeze-dried in 5.5 ml aliquots.

Testing of Lot 1-81 (TSI-GSD 210) is summarized in Table 14 . The two drying runs yielded vaccine with good potency.

IV. Certification of WEE Vaccine Seed Virus

The passage history of the CM4884 WEE virus is outlined in Table 15 . The chick embryo tissue suspension virus has been identified as WEE using a known specific antiserum against the B-11 virus strain. The seed virus is free of bacterial contamination.

A test for possible adventitious agents is in progress.

V. Preparation of WEE Virus Antiserum

A known tissue culture preparation of the B-11 strain of WEE virus was inoculated into suckling mice (3-5 days) IC and the infected brain tissue harvested at 48 hours. The 10 percent triturated tissue suspension was inoculated into 12 rabbits by the IM route (0.5 ml in each thigh). Two doses were given at a two week interval and the rabbits were exsanguinated three weeks later.

The pooled rabbit sera was filtered and freeze-dried in 0.5 ml aliquots. Repeated tests in Vero and CEC tissue cultures failed to show the presence of live virus in the reconstituted antiserum. The antisera will be used to attempt neutralization of the seed virus in a search for adventitious agents.

The hemagglutinin inhibition titer of the dried antiserum is 1:1600. The plaque reduction neutralization titer is 1:2048 (80% end point).

Table 13

Comparison of Three WEE Viruses as Candidates for Vaccine Seeds

Virus Isolate	Passage No.[1]	Passage Host	Growth Time (hrs.)	Growth CPE (%)	Titer (log_{10})[2] PFU/0.5 ml	ED_{50},ml[3]
1. #77500	2	CEC	36	10	6.98[4]	--
	3	CEC	25	60	7.90	--
	4	CEC	24	10	7.75	0.42
2. #775944	2	CEC	44	20	8.10[4]	--
	3	CEC	31	30	7.04	--
	4	CEC	24	10	7.81	>0.50
3. #CM-684	2	CEC	44	10	8.19[4]	--
	3	CEC	25	60	8.64	--
	4	CEC	24	10	6.95	>0.50
	3[5]	Egg	24	--	10.73	--
	4	CEC	24	60	9.87	0.02

[1] Passage 2 initiated with first pass CEC tissue culture fluid received from USAMRIID.

[2] Titration done in CEC by plaque method.

[3] ED_{50} determined in weanling mice that received two doses of vaccine IP (0.5 ml/dose) and challenged with live WEE virus, strain B-11, IC.

[4] Titer of the terminal dilution fluid. For seeds 1 and 2 this was 10^{-9}, seed 3 was 10^{-7}.

[5] 8-day embryonated eggs inoculated in the yolk sac with the terminal dilution of pass 2 in CEC. The titer given is that for an embryo suspension from eggs that received 10^{-2} dilution of pass 2.

Table 14

Status of WEE Inactivated Vaccine

(Lot 1-81) TSI-GSD 210

Test	Result
Bulk vaccine	
Bulk sterility	Sterile
PPLO	Negative
TB (Lowenstein-Jensen)	Negative
Two week hold-control cultures	Normal
Hemadsorption-control cultures	Negative
Infectivity: CEC	$10^{-9.5}$/ml
Suckling mice	$10^{-10.0}$/ml
Potency (g. pig - ml ED_{50})	0.008[1]
Residual live virus (suckling mice)	Negative
Freeze-dried vaccine (2 runs)	
Bulk sterility	Sterile
Final container sterility	Sterile
General safety (g. pig/mouse)	Satisfactory
pH	6.9
Residual formalin	0.006% (Run 1) 0.012% (Run 2)
Residual moisture	0.34% (Run 1) 0.29% (Run 2)
Potency (ED_{50},ml)	0.005 (Run 1) 0.004 (Run 2)

[1] Lot 2-74 USAMRIID was 0.008 for comparison.

Table 15

Passage History of WEE Vaccine Seed Virus, CM4884

Passage	Host	By
Isolation	Mosquito Pools	Fort Collins
1	Certified CEC (Pool 28)	USAMRIID
2	CEC (SPAFAS MR-63)	TSI-GSD
3	20% Chick Embryo Suspension (SPAFAS LEB-6)	TSI-GSD*
4	Lot 1-81 Vaccine	TSI-GSD

*Tests to certify vaccine seed:

TB cultural - Negative

Sterility - Negative

Identity - Positive for WEE virus

Adventitious Agents - In process

WEE Vaccine Potency Testing

I. Introduction

Lot 1-81 (TSI-bulk-liquid), Lot 2-74 (USAMRIID-final product) and Runs 1 and 2 of Lot 1-81 (TSI-GSD 210) WEE vaccine were potency tested in guinea pigs by HI and protection followed by challenge.

II. Potency Test Results

Two WEE vaccines were potency tested, Lot 1-81 (TSI-bulk-liquid) and Lot 2-74 (USAMRIID-final product). The results in Table 16 show that the two vaccines were equivalent by all criteria. The survivors at all dilutions of the vaccine were about the same. All animals tested by HI converted serologically and the ED_{50} by the Reed & Muench gave identical values of 0.008 ml.

Results of potency testing Lot 1-81 Runs 1 and 2 (TSI-GSD 210) are shown in Table 14 with values of 0.005 for Run 1 and 0.004 for Run 2. These two runs have been freeze-dried and constitute the final product.

Table 16

WEE Vaccine Potency Testing

WEE Vaccine	Survivors/Total Immunized with Vaccine Dilutions at 1:5	1:25	1:50	1:100	1:200	Serological Conversion by HI from Sera of Guinea Pigs Given Vaccines Diluted 1:5	1:25	PSM Dose
Lot 1-41 (TSI) (Bulk liquid)	10/10	9/9	3/5	1/6	0/6	10/10	10/10	0.008 ml
Lot [illegible]-74 (USAMRIID) (Final product-freeze-dried)	10/10	10/10	4/6	0/6	0/6	10/10	10/10	0.00[illegible] ml

Rift Valley Fever Vaccine (WRAIR)

Potency Testing

I. Introduction

Lots 18 and 19 of Rift Valley Fever Vaccine prepared during June 1964 at WRAIR were potency tested in accordance with instructions contained in the 26 March 1981 letter from WRAIR.

II. Potency Test Results

Potency test results revealed that Lot 18 failed the potency requirements established as 0.02 ED_{50} since it had a potency value of 0.083. Lots 19 and the reference vaccine (Lot 12-2, TSI), on the other hand, passed potency with values of 0.011 and 0.004, respectively. The challenge virus contained 6000 $MIPLD_{50}$/ml and the reference vaccine gave a value similar to those obtained in prior tests (0.004 to 0.007 ml ED_{50}).

Rift Valley Fever (RVF) Vaccine Prepared at The Agouza Institute

I. Introduction

Rift Valley Fever Vaccine, Lots 52, 53, 60 and 63, prepared by The Agouza Institute, Cairo, Egypt was tested and a report was submitted to USAMRIID.

II. Testing

Four lots of RVF vaccine prepared by The Agouza Institute were tested for potency and adventitious agents in our laboratory. An original and revised report were submitted to USAMRIID.

An undentified agent causing CPE in Vero cells was found in lots 53 and 60. These two lots had been freeze-dried.

This unknown agent was sent to Dr. Shope at the Yale Arbovirus Research Unit and Dr. Nakano at the Centers for Disease Control, Atlanta, Georgia.

III. Results

Dr. Nakano identified the extraneous virus, detected in the Agouza RVF vaccine, as vaccinia. Although no viral particles were detectable by electron microscopy, 12 day old embryonic chicken CAMS inoculated with cell culture fluid revealed typical vaccinia pocks that titered [illegible] pock forming units/0.1 ml.

Q Fever Vaccine Stability Testing

I. Introduction

Lots 2 and 4 of Q Fever Vaccine (NDBR 105) were potency tested after storage at -20°C for nine years.

II. Potency Test Results

Potency tests performed on Lots 2 and 4 of Q Fever Vaccine (NDBR 105) were completed.

Test results show that the two lots have potency values that have not decreased during nine years storage at -20°C (see Table 17).

Table 17

Q Fever Vaccine Storage Stability Potency Test Results

(NDBR 105)

Lot No.	1972	1981
2	0.23*	0.23
4	0.89	0.23

*μgms/ml of vaccine required to convert serologically 50% of the vaccinated guinea pigs.

Attenuated Chikungunya Vaccine
Safety Test in Rabbits and Mice

I. Introduction

In accordance with instructions received from USAMRIID October 22, 1980 and January 5, 1981 safety tests on attenuated Chikungunya virus were performed in rabbits and mice, the directive received from USAMRIID June 12, 1981 and July 17, 1981 required safety tests to be done in suckling mice only.

II. Results

Safety Tests Performed in Rabbits and Mice During the First Quarter of 1981

A. Rabbit Test

Although one of the ten rabbits on test died two days after receiving the vaccine, this animal died from acute colitis as ascertained by gross and microscopic pathology and "it is unlikely that the vaccine had a direct or indirect causal relationship to the colitis" as determined by the pathologist. The rabbit test passed requirements of 21 CFR 630.16 (a) (1) dated April 1, 1980 and a detailed report was sent to USAMRIID January 22, 1981.

B. Mouse Test

Although all sixty of the adult mice survived the safety test, 48 of 55 suckling mice participating in the test died. A suspension from carcasses of the suckling mice was capable of producing CPE in Vero cells. A detailed report was submitted to USAMRIID March 27, 1981 noting that the vaccine under test did not meet the requirements of 21 CFR 630.15 (a) (3) dated April 1, 1980.

Safety Tests Performed in Suckling Mice During the Third Quarter of 1981

A. Suckling Mouse Test

Production Chikungunya seed (28 May 1981) and Control for production seed (Chik) passed the unofficial safety est. There was only sufficient material available to inoculate 13 suckling mice with Production Chikungunya seed (28 May 1981) and 5 suckling mice with Control for production seed (Chik). The test mice survived the two week observation period with the exception of two animals receiving the Production seed (28 May 1981) that were cannibalized immediately after inoculation.

Chikungunya 181 Pass 1 dated 7/10/81 and 181 Outgrowth Lot 2 72 hrs 10/19/79 passed safety testing in suckling mice. In performance of this test each of two groups of twenty 24 hour suckling mice was inoculated, 0.01 ml ic and 0.1 ml ip per mouse, with the above viruses. All 40 suckling mice involved in the test survived the 14 day observation period.

Tularemia Vaccine Stability Studies

I. Introduction

Tularemia Vaccine (NDBR 101) Lots 2, 4 and 9 stored at -20°C for at least 17 years were potency tested.

II. Potency Test Results

Testing showed no significant differences from previous values obtained with these three test vaccines. Tests included viable count, mouse virulence and mouse protection. Results summarized in Table 18 give values that fall within limits prescribed for the tests.

Table 18

Pasteurella Tularensis Vaccine, Live
Storage Stability Testing
(NDBR 101)

Lot No.	Viable Count (per ml)	Mouse Virulence (%)	Mouse Protection (%)	Date of Preparation
2	2.5×10^9	6.6	98	May 1962
4	3.0×10^9	20.0	99	May 1962
9	3.0×10^9	12.5	93	June 1964

RVF Vaccine Development

I. Introduction

At the request of USAMRIID three strains of RVF were compared for growth in five cells available in this laboratory. Each of the viruses yielded similar titers in all cell lines, however the Entebbe virus harvest had the lowest titer and the ZZ501 virus the highest, the SA-75 was intermediate.

The ZZ501 RVF virus was passed in certified FRhL-2 cells for a production seed and testing is in progress to certify the seed for vaccine preparation.

II. Growth Curve Experiments

Two experiments were done in essentially the same manner. The viruses were diluted and inoculated on to the cells so that the ratio of virus: cell was less than 1:10. The actual MOI ratios from 1:100 to 9:100 (except ZZ501 with CER-4 cells which was 10-fold lower) depending on the cell growth. Maintenance medium was Earles basal medium supplemented with 5 gm/l of bovine albumin, fraction V. The medium overlay was sampled at regular intervals from 4 to 70 hours post-infection. Virus samples were titrated in BHK-21 cells to estimate number of plaque-forming units shed into the medium.

A comparison of growth of Entebbe and SA-75 in MRC-5, CER4, FRhL-2 and BHK-21 cells is shown in Table 19 . Entebbe yielded best titers in MRC-5, BHK-21 and FRhL-2 (6.6 - 6.7) with only 5.5 in CER4 cells. SA-75 yielded the best titers in FRhL-2 (8.6) with a ten-fold lower titer in the other three cells.

In a second study, ZZ501 was evaluated in Vero, CER4, FRhL-2 and BHK-21 cells. There was little difference in the peak titers achieved (8.4 - 8.7) with varying times of peak titer of 38 hours for BHK-21 to 62 hours for Vero and CER4 cells. FRhL-2 cells yielded peak virus yield at 46 hours for both SA-75 and ZZ501, while Entebbe took 54 hours.

III. Preparation of the ZZ501 Seed Virus

The passage history of this virus is:

Human Serum

↓

P1 Certified FRhL-2 Cells

At The Salk Institute a second passage of this virus was made in

FRhL-2 cells with Earles basal medium containing 2% human serum albumin. Virus was harvested at 45 hours. The virus harvest was sterile and had a titer of 8.3 $\log_{10}$ in BHK-21 cells. Additional testing is in progress to certify this seed for vaccine production.

Table 19

Growth of Entebbe and SA-75 Strains of RVF Viruses in Four Cell Lines

Time-hrs. Post-infection (MOI)	Entebbe				SA-75			
	MRC-5 0.07	CER-4 0.07	FRhL-2 0.08	BHK-21 0.02	MRC-5 0.07	CER-4 0.09	FRhL-2 0.02	BHK-21 0.02
	(PFU/ml, $\log_{10}$)							
4	2.5	3.9	3.0	2.3	2.3	1.0	2.8	1.0
14	4.9	4.5	4.5	4.5	6.0	4.6	4.7	4.3
22	4.9	4.5	5.2	5.3	6.7	5.9	6.9	5.9
30	5.3	5.0	5.5	5.7	6.5	6.6	7.4	6.1
38	5.6	5.2	5.2	6.7 (60%)	7.4	6.7	7.6	7.1
46	6.4	5.4	6.1	6.4	7.7 (10%)	6.5	8.6 (100%)	7.1
54	6.5	5.5 (60%)	6.6 (60%)	6.4	7.0	7.2 (100%)	8.0	7.6 (100%)
62	6.7 (50%)	5.5	6.1	6.6	7.7	7.2	8.3	7.5
70	6.5	5.3	6.5	6.3	8.3	7.2	8.1	7.3

(%) = estimated percent of CPE at peak titer.

Table 20

Growth of Zagazig #501 Strain of RVF Virus in Four Cell Lines

Time-hrs. Post-infection (MOI)	Vero 0.01	CER-4 0.002	FRhL-2 0.02	BHK-21 0.01
		(PFU/ml, $\log_{10}$)		
4	2.7	2.6	2.5	3.7
14	4.5	3.7	4.2	5.0
22	5.8	5.7	6.1	6.2
30	7.4	7.2	7.2	7.6
38	7.7	8.2	8.1	<u>8.6</u> (5%)
46	8.2	8.2	<u>8.5</u> (15%)	8.5
54	8.2	8.4	8.4	8.3
62	<u>8.4</u> (70%)	<u>8.7</u> (100%)	7.9	8.6
70	8.1	8.4	8.5	8.5

(%) = estimated percent of CPE at peak titer.

RVF Vaccine Potency Testing

I. Introduction

The potency test described in this report was performed in accordance with instructions received from USAMRIID and differs from RVF vaccine potency tests previously conducted at TSI in the following respects; the Zagazig 501 strain replaced the Entebbe strain as challenge virus, 42-day-old mice were used, and one dose of vaccine was administered instead of two doses.

II. Potency Test Results

Three lots of RVF vaccine (TSI-GSD 200) were potency tested. The 50 percent effective dose determinations by probit analysis and the Reed & Muench procedure are shown in Table 21 and compared to previously reported results. The one dose mouse test differentiates Lot 2-2 from Lot 3-1 but not from Lot 4-2. This is in contradistinction to results previously reported for the two-dose mouse test and the human serologic response as reported by Meadors.

The virus, Zagazig 501, used in the test system had an LD_{50} of 1000 per challenge dose of 0.1 ml given intraperitoneally.

Table 21

Mouse Potency Tests on RVF Vaccine (TSI-GSD 200) vs.
Human Response[1]

	Mouse Protection Tests				Human Response	
	one-dose			2-dose	PRNT80	
	Probit Analysis		R&M	R&M	Geometric $\bar{x}$ Titer	
Lot-Run	Day 10	Day 18	Day 18	Day 21	Day 42	n=
2-2	0.004[2]	0.008	0.012	0.004	1:160	5
3-1	0.013	0.019	0.025	0.005	1:48	4
4-2	0.004	0.011	0.015	0.008	1:806	3

[1] One dose mouse protection tests in 42 day old mouse, two dose tests in 21 day weanlings. Human response data drawn from Meadors as reported by Peters (memorandum to the Commander USAMRIID, 12 November 81, Table 1a, page 9).

[2] ED_{50} (ml of vaccine).

Rift Valley Fever Antisera

I. Introduction

Rabbit antisera were produced against inactivated Zagazig and H1849 strains of RVF virus.

Rabbit antisera (TSI-GSD 201 Lot 3) were also prepared using live, Entebbe strain, RVF virus as the immunogen.

II. Antisera Production

A. Inactivated Zagazig and H1849 (RVF) Strains

Formalin inactivated vaccines prepared from Zagazig and H1849 RVF viruses were used as immunogens in producing these antisera. Both viruses were isolated from human sera and passed twice in FRhL-2 cells. The second passage in FRhL-2 cells was used in vaccine preparation. Multiple doses of vaccine given iv were used to immunize the animals prior to exsanguination. Both antisera passed sterility testing and had acceptable HI titers. A shipment made to USAMRIID consisted of 70 x 0.5 ml of Zagazig and 140 x 0.5 ml of H1849 (RVF) rabbit antisera.

B. Live Entebbe (RVF) Strain

Live Entebbe strain (RVF seed #185-A) was used to prepare antisera. Rabbits were given 0.1 ml intradermally at each of two sites and 0.2 ml subcutaneously at one site. Four weeks following inoculation the rabbits were exsanguinated by cardiac puncture. After processing, the sera passed sterility, potency testing by HI and examination for residual live virus.

Dr. Shope at Yale University received 50 x 0.5 ml vials and the remainder of the lot (TSI-GSD 201 Lot 3) consisting of 425 x 0.5 ml vials was sent to USAMRIID.

A detailed protocol for production of the RVF antiserum in rabbits was sent to USAMRIID September 11, 1981.

HA Antigens

I. Introduction

One lot of EEE, Lot 1-81, HA antigen was prepared and dried and one lot of WEE HA antigen (Lot 1-81) was dried. Safety testing in suckling mice remains to be done. A total of 504 amps of RVF HA antigen (Lot 2-80) was shipped to Dr. Shope.

II. Processing

A. EEE Antigen, PE6 (sucrose-acetone extracted suckling mouse brain)

This lot was dried yielding 358 vials. A dilution of 1:100 yields 4 units in the HI test.

B. WEE HA Antigen, B11 (sucrose-acetone extracted suckling mouse brain)

Freeze-drying of this lot was completed yielding 504 vials as Lot 1-81 with a titer of 1:1024. A total of 216 amps of Lot A-72 was shipped to USAMRIID.

C. RVF HA Antigen, Entebbe (sucrose-acetone extracted suckling mouse liver)

Five hundred and four (504) vials of Lot 2-80 were shipped to Dr. Shope.

D. WEE HA Antigen

Shipment of 216 amps of Lot 1-80 was made to USAMRIID.

III. HA Antigen Inventory

The inventory and status is given as Table 22.

Table [illegible]

[illegible] Freeze-Dried, [illegible] Extracted, PEL Inactivated, Hemagglutination-Inhibition Test Antigens

[illegible] No.	Description	Lot No.	Date Dried	HA Titer	No. Vials	Safety Tested: Vero	Safety Tested: Suckling Mice
[illegible]	[illegible] Suckling Mouse Liver [illegible] Strain	1-[illegible]	[illegible]/1/79	1:64 1:512 (Original)	62	Yes	Yes
[illegible]	" " " "	3-7[illegible]	10/11/79	1:32 1:512 (Original)	178	Yes	Yes
[illegible]	" " " "	1-80	2/7/80	1:64 1:1024 (Original)	55	Yes	Yes
[illegible]	" " " "	2-80	[illegible]/4/81	1:128 1:1024 (Original)	1989	Yes	Yes
[illegible]	" " " "	1-81	[illegible]/11/81	1:128 1:1024 (Original)	468	Yes	Yes
[illegible]	EEE-Suckling Mouse Brain [illegible] Strain	1-80	1/20/81	1:1024	69	Yes	Yes
[illegible]	" " " "	1-81	[illegible]/9/81	1:2560	355	Yes	Yes
[illegible]	WEE-Suckling Mouse Brain B-11 Strain	A-72	3/26/81	1:256	62	Yes	Yes
[illegible]	" " " "	1-81	8/5/81	1:2048	501	Yes	Yes
[illegible]	VEE-Suckling Mouse Brain Trinidad Strain	1-80	2/18/81	1:1024	156	Yes	Yes
[illegible]	Chikungunya-Suckling Mouse Brain-Strain 168	1-80	2/18/81	1:256	155	Yes	Yes
---	Normal Mouse Liver Antigen	24-7[illegible]	3/27/81	------	418	Yes	ND*

*[illegible]

Dengue Antiserum

I. Introduction

Dengue, type 1 virus, Hawaii strain, received from Dr. Shope at the Yale Arbovirus Research Unit was passed in suckling mice. The infected mouse brain material served as a live immunizing agent that was then injected into rabbits. Sera obtained from inoculated rabbits passed our testing procedures and were dispensed.

II. Antiserum Production

Dengue, type 1 virus, Hawaii strain, mouse pass 17 obtained from Dr. Shope was passed in suckling mice by inoculating them with 0.01 ml intracerebrally. One week later a 10% suspension, prepared from brains collected from moribund mice, was tested and titered $10^{-7.2}$ per ml when injected intracerebrally in 3-week-old mice. This suspension containing live Dengue virus was then used to inoculate each of 6 rabbits with 0.1 ml intradermally at each of 2 sites, 0.2 ml intramuscularly at one site and 0.2 ml subcutaneously at one site. Four weeks after inoculation, sera samples were obtained from the rabbits and tested by LHI. Sera from 4 of the rabbits had sufficiently high titers to warrant their use. The pooled sera passed sterility and the test for residual live virus. A total of 434 x 0.4 ml vials (frozen) designated as TSI-GSD 211, Lot No. 1 is on hand.

Drug Screening Program

I. Introduction

Virus stocks were prepared and assays established for the four viruses to be used in drug screening. Mouse protection tests (MPT) were performed against RVF (Entebbe strain) virus with 13 of 15 drugs received from USAMRIID. One MPT was conducted against VEE (Trinidad strain) virus using Ly 122771. Plaque reduction tests (PRT) were completed using RVF virus and 15 drugs. The same test was used to test 21 drugs against VEE virus and 13 drugs against Pichinde virus. One yield inhibition test (YIT) was performed using Atromid-S against RVF virus. The OCT-541 strain of JBE virus was standardized.

II. Results

A. Virus Stocks

Virus stocks were prepared against the Trinidad strain of VEE, Entebbe strain of RVF, Pichinde and the OCT-541 strain of JBE viruses. Assays were then established using these four viruses.

B. In vivo Testing (MPT)

1. RVF Virus

Results of mouse protection tests using RVF (Entebbe strain) as the test virus against 13 of the 15 drugs received from USAMRIID are shown in Table 23 . Only enough material was received to perform *in vitro* tests on drugs RK-15 and RKR-244. They, therefore, will be excluded from *in vivo* testing. Since, Nafenopin (Ciba) had been reported to be toxic for mice at 100 mgms we decreased the dosage to 20 mgms. The drug was neither toxic nor effective at this concentration. Pyrazofurin (Lilly) and AR 20613 were the only drugs to exhibit toxicity for mice, the former at 100 and 25 mgms and the latter only at 100 mgms. The mice treated with the remaining drugs showed no deaths from toxicity at 100 mgms. Although Atromid-S was not toxic for mice at 100 mgms, four unexpected deaths occurred in toxicity controls given 25 mgms of the drug two days after the course of treatment was completed.

The use of RA 114 caused a 10 day delay of death after challenge in two of ten mice treated with 100 mgms of the drug and challenged with >1000 LD_{50}'s of RVF-185A. All 10 mice given 25 mgms of RA 114 during treatment died 3 days post challenge. RA84 had 20% survivals and delay of death in the remaining mice @100 mgms. RA-98 showed a delay of death. The most promising drug tested was Ly 122771/2 since all mice (controls and

challenged) given either 100 or 25 mgms survived the 3 week observation period. These results were fortified by testing the drug against RVF by plaque reduction and noting its effectiveness (Table 24).

2. VEE Virus

Due to the paucity of the test drugs only one mouse protection test was performed. This exception was made with drug Ly 122771 because of its performance against RVF virus in both the MPT and PRT. The results were disappointing since the drug was ineffective against VEE; all treated mice given VEE died on the same day as the animals in the back titration receiving the challenge dose.

C. In vitro Testing (PRT)

1. RVF Virus

All 15 drugs were tested against RVF by PRT. Of these only two demonstrated possible toxicity at the 500 μgm level; Pyrazofurin, reported previously as being toxic in mice, and Ly 122771 that resulted in equivocal toxicity.

Table 24 shows the % reduction of RVF plaques at various concentrations of drugs. The most noteworthy drugs tested were Pyrazofurin (Lilly), AR 20613 and Ly 122771 (Lilly). The first two candidates inhibited plaques 100% @500 μgms and approximately 35% @100 μgms. The latter, which was also effective in protecting mice (Table 23) was by far the most promising drug tested to date by PRT with 100% reduction @25 μgm concentration (Table 24).

2. VEE Virus

All 21 drugs were tested against VEE by PRT (Table 25). Drug RA197 was the only drug to show possible toxicity at the 500 μgm level. Eight of the drugs, totally ineffective, showed no reduction of plaques @500 μgms. Eleven of the drugs that showed some effectiveness against VEE reduced plaques from 20% to 100% at the 500 μgm level. The two most effective drugs against VEE in the PRT, Atromid-S and RKR-244, were able to reduce plaques 100% @250 μgms and 41% @10 μgms with the first drug and 100% @250 μgms and 32% @100 μgms with the latter.

3. Pichinde Virus

The thirteen drugs shown in Table 26 were tested against Pichinde virus this year. In this series of tests only RB108 showed toxicity at 500 μgms. The three drugs that showed the most promise against

Pichinde virus in the PRN were AR 20613 (WRAIR) with reduction in plaques of 100% @500 μgms and 50% @100 μgms, Ly 122771 (Lilly) with 91% reduction @500 μgms and 70% @250 μgms and Ribavirin with 100% reduction @250 μgms and 52% @100 μgms. Four other drugs showed plaque reductions to lesser degree and six drugs showed no reduction.

C. In vitro Testing (YIT)

1. RVF Virus

Atromid-S, the only drug tested by the YIT, inhibited 57% of the plaques @500 μgms and none @100 μgms.

D. Miscellaneous

1. The OCT-541 strain of JBE virus was entered in the drug screening program as a Flavivirus representative near the end of this year. Standardization tests were performed with Ribavirin.

Table 23

Drug Screening Program

<u>In vivo</u> Mouse Protection Test-RVF

Drug	Delay to Death	Toxicity	Solubility	Comments
Nafenopin (Ciba)	@20 mgms No delay of death	Not toxic @20 mgms	Added NaOH to solubilize pH 6.0-8.0	
Atromid-S (Ayerst)	No delay of death	Not toxic @100 mgms	Added NaOH to solubilize. pH 6.4-7.0	4 mice given 25 mgms on the toxicity control died 2 days following treatment with the drug.
Licrysone (KK)	No delay of death	Not toxic	Soluble @pH 7.8 + heat	
Pyrazofurin (Lilly)	Deaths from toxicity	Toxic @100 & 25 mgms	Very soluble	
LY 122771 (Lilly)	No deaths	Not toxic	Soluble but milky	Repeat test if drug available All animals died on 2nd challenge.
RB-108	No delay of death	Not toxic	Crystals formed. Soluble upon heating.	
RK-15	Not available	----------		
RB-98	No delay of death	Not toxic	Crystals formed. Soluble upon heating.	
RKR-244	Not available	----------		
PV-33	No delay of death	Not toxic	Soluble in HOH.	
AR 20613	No delay of death	Toxic @100 mgms	Very soluble.	
RA-114	Delay of death	Not toxic @100 mgms	Very soluble.	Mice with 25 mgms died 3 days with 100 mgms 2 delayed 10 days.
RA-83	No delay of death	Not toxic @100 mgms	Soluble	
RA-84	20% survival and delay of death	Not toxic @100 mgms	Soluble	
RA-98	Delay of death	Not toxic @100 mgms	Soluble	

Table 24

Drug Screening Program
In vitro Plaque Reduction Test - RVF

Drug	% Reduction	Toxicity
Nafenopin (Ciba)	62% @500 μgms	Not toxic
Atromid-S (Ayerst)	38% @500 μgms	Not toxic
Licrysone (KK)	73% @500 μgms 34% @ 25 μgms	Not toxic
Pyrazofurin (Lilly)	100% @500 μgms 35% @100 μgms	Toxic @500 μgms Not toxic @250 μgms
Ly122771 (Lilly)	100% @ 25 μgms 68% @ 10 μgms	+/- toxicity @500 μgms
RB-108	56% @500 μgms	Not toxic
RK-15	*No reduction @500 μgms	Not toxic
RP-98	50% @500 μgms	Not toxic
RKR-244	*22% @500 μgms	Not toxic
RV-33	29% @500 μgms	Not toxic
AR20613	100% @500 μgms 36% @ 25 μgms	Not toxic
RA-114	25% @500 μgms	Not toxic
RV 202	*No reduction @500 μgms	Not toxic
RA 197	*No reduction @500 μgms	Not toxic
Ribavirin	50% @500 μgms	Not toxic

*Drug added one hour before virus.

Table 25

Drug Screening Program

In vitro Plaque Reduction Test - VEE

Drug	% Reduction	Toxicity
Nafenopin (Ciba)	66% @500 μgms 58% @250 μgms	Not toxic
Atromid-S (Ayerst)	100% @250 μgms 41% @ 10 μgms	Not toxic
Licrysone (KK)	100% @500 μgms 50% @ 10 μgms	Not toxic
Pyrazofurin (Lilly)	No reduction @500 μgms	Not toxic
Ly122771 (Lilly)	76% @500 μgms 48% @250 μgms	Not toxic
AR20613 (WR)	61% @500 μgms 51% @250 μgms	Not toxic
RA-114	No reduction @500 μgms	Not toxic
RV-33	No reduction @500 μgms	Not toxic
RB-38	No reduction @500 μgms	Not toxic
RA-116	No reduction @500 μgms	Not toxic
RA-102	100% @500 μgms 72% @250 μgms	Not toxic
RV-202	20% @500 μgms	Not toxic
RV-2[illegible]3	36% @500 μgms 30% @250 μgms	Not toxic
NSC 137679	89% @500 μgms 28% @ 10 μgms	Not toxic
RA 1[illegible]7	52% @250 μgms 26% @ 10 μgms	Toxic @500 μgms
RA 1[illegible]8	No reduction @500 μgms	Not toxic
RB [illegible]08	No reduction @500 μgms	Not toxic
RK 15	No reduction @500 μgms	Not toxic
RKR 244	100% @250 μgms 32% @100 μgms	Not toxic

All drugs added one hour before virus.

Continued ------

Table 25 (continued)

Drug Screening Program
In vitro Plaque Reduction Test - VEE

Drug	% Reduction	Toxicity
Ribavirin	62% @500 μgms 29% @ 25 μgms	Not toxic
RA-98	26% @500 μgms 15% @100 μgms	Not toxic

All drugs added one hour before virus.

Table 26

Drug Screening Program

In vitro Plaque Reduction Test* - Pichinde

Drug	% Reduction	Toxicity
Nafenopin (Ciba)	48% @250 μgms 29% @ 25 μgms	Not toxic
Atromid-S (Ayerst)	No reduction @250 μgms	Not toxic
Licrysone (KK	No reduction @500 μgms	Not toxic
Pyrozofurin ILilly)	66% @250 μgms 26% @100 μgms	Not toxic
Ly122771 (Lilly)	91% @500 μgms 70% @250 μgms	Not toxic
AR20613 (WR)	100% @500 μgms 51% @100 μgms	Not toxic
RA-114	No reduction @500 μgms	Not toxic
FV-33	No reduction @500 μgms	Not toxic
PB-98	No reduction @500 μgms	Not toxic
RB 108	12% @100 μgms	Toxic @500 μgms
RK 15	58% @500 μgms 21% @250 μgms	Not toxic
RKR 244	No reduction @500 μgms	Not toxic
Ribavirin	100% @250 μgms 32% @100 μgms	Not toxic

*All drugs added one hour before virus.

Korean Hemorrhagic Fever

(G.R. French)

I. Introduction

A KHF laboratory was established this year in Room E Suite III with the installation and final testing of a Class II Type A Nu-Aire Laminar Flow Biological Safety Cabinet with air thimble that exhausts via the building exhaust air system. The usual problems were encountered in getting this cabinet to meet design specifications due to the enormous air movement requirements. Adequate negative pressure in the air shafts exist, however the shafts are not designed to carry this large a volume of air and successful installation required several days of trial and error balancing of air in the remainder of the suite. Our experience would indicate that installation of more than one cabinet per suite that exhausts to the building air is not practical, and further installations of this type in other suites should be preceeded by replacement of the existing 6" diameter branch shafts with 12" shafts that can adequately carry the required volume of air. Replacement of the air shafts will require penetration of the reinforced concrete slab between the laboratory room and the interstitial space.

Production of diagnostic reagents in support of the KHF program began early in October and continues to date as indicated below. The one lot of rabbit antiserum prepared was produced with upgraded hot suite discipline, i.e. restricted entry of the area, Hepa filter masks, caps, gowns, gloves and plastic boots which were donned prior to entry of the animal room and removed during exit. Masks were "sanitized" between entries by exposure to ozone and ultraviolet light in the pass through box. Discarded outerwear, rabbit excreta, and exsanguinated animals were bagged and retained in the animal room until paraformaldehyde treatment at the completion of the experiment. There were no unusual occurrences during the course of the experiment.

II. Diagnostic Reagents Produced

25 ten-spot FA slides Reovirus type 1 lot 1
25 ten-spot FA slides Reovirus type 2 lot 1
25 ten-spot FA slides Reovirus type 3 lot 1
50 ten-spot FA slides Reovirus polyvalent types 1,2,3 lot 1
25 ten-spot FA slides Reovirus type 1 lot 2
25 ten-spot FA slides Reovirus type 2 lot 2
25 ten-spot FA slides Reovirus type 3 lot 2

250 ten-spot FA slides Reovirus polyvalent types 1,2,3 lot 2
300 ten-spot FA slides Hantaan virus strain 76-118 A549 P-15 Vero P2 lot 1
300 ten-spot FA slides Hantaan virus strain 76-118 A549 P-15 Vero P2 lot 2
150 ten-spot FA slides Hantaan virus strain 76-118 A549 P-15 Vero P2 lot 3
150 ten-spot FA slides Hantaan virus strain 76-118 A549 P-17 lot 4
190 ml Rabbit antisera to Hantaan virus strain 76-118 A549 P-15 lot 1 pool 1*
98 ml Rabbit antisera to Hantaan virus strain 76-118 A549 P-15 lot 1 pool 2*

*Pool 1 titers 1:1280 vs Hantaan virus FA slides and 1:160 vs Reovirus polyvalent FA slides. Pool 2 titers 1:1280 vs Hantaan virus FA slides and ≤1:20 vs Reovirus polyvalent FA slides.

Immunization Program

I. Introduction

Seven new employees were immunized with Q Fever, RVF, EEE, VEE and WEE vaccines and six received Tularemia Vaccine.

II. Results

Seven new employees (one TSI and six Connaught personnel who require access to infectious areas) were given the 0.1 ml test dose of Q Fever Vaccine. One employee who reacted with induration and erythema was dropped from receiving the Q Fever series. The remaining six subjects completed the full series of 3 doses.

All new candidates, except for the Q Fever drop out and one employee not present during immunization against Tularemia, completed the full course of immunizations.

Five of the 7 employees had significant levels of protective antibodies against VEE as measured by PRN.

All seven had acceptable titers against RVF by PRN.

Distribution

5 copies	Commander U.S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Frederick, MD 21701
4 copies	Commander U.S. Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, MD 21701
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDA Cameron Station Alexandria, VA 22314
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20014
1 copy	Commandant Academy of Health Sciences, U.S. Army ATTN: AHS-CDM Fort Sam Houston, TX 78234
1 copy	Director Walter Reed Army Institute of Research Walter Reed Army Medical Center ATTN: SGRD-UWZ-C Washington, DC 20012

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