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REPLY TO ATTENTION OF

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MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Mrs. Judy Pawlus at DSN [redacted].

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for CORNELIUS R. FAY III
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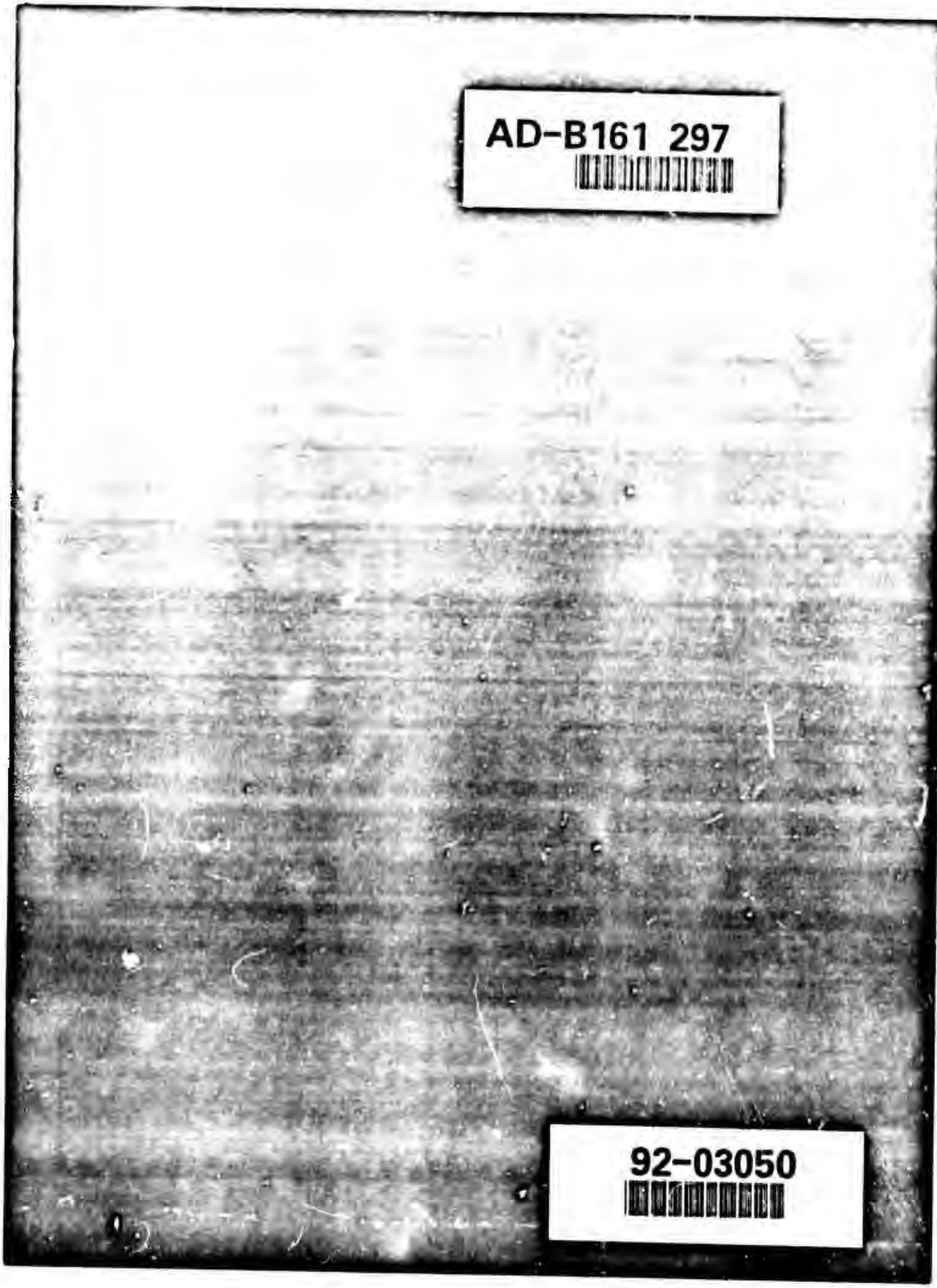

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Ninety-three drugs were tested against LCMV in the adult mouse model. Fourteen drugs gave VR scores >1.2 which places these drugs in the top 90th percentile of all drugs tested in the LCMV model.

The identity of the ribavirin resistant virus has been established by mouse neutralization test using acetone-ether extracted CCHF virus mouse polyclonal antibody. There were no breakthrough mice in the neutralization test. Ribavirin-resistant virus was re-isolated from liver tissue of infected mice.

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After infection of mice with vaccinia virus, tail lesions could be easily quantitated by staining tails with a mixture of methylene blue and fluorescein.

Each of 5 virus strains was grown in Vero cells and titrated by plaque formation. The lowest plaque titer was obtained with the wild type (398 PFU/0.1 mls), the highest with the Copenhagen strain (630,957 PFU/0.1 mls). The plasmid and 3PP strains had identical plaque titers, 25,199 PFU/0.1 mls. Mice were inoculated with the lowest possible dose, 398 PFU of each strain. Lesions were counted. The wild type was the most virulent strain requiring only 9 PFU to produce a lesion. The Lister, 3PP, and Copenhagen were the next more virulent in that order with 66, 284, and 995 PFU, respectively, required to produce lesions. The plasmid strain did not produce lesions with this inoculum dose .

The experiment was repeated using the maximum number of PFUs for each virus as inoculum. The results were generally similar. The most virulent virus strain was the wild type which required only 9 PFU to produce a tail lesion. The Lister, Copenhagen and Plasmid strains were the next most virulent in that order requiring 249, 7,336, and 17,942 PFU, respectively, to produce a single lesion. Using a high virus dose inoculum, the 3PP strain required 62,797 PFU to produce a lesion.

Two inbred mouse strains, BALB/C and NU/NU, and two random bred mouse strains CF-1 and CD-1 were inoculated with the Copenhagen strain of vaccinia virus. Lesions were counted immediately after formation (days 6-7) and later just before the lesions were resolved (days 8-10) depending upon the individual mouse strain. The variation about the mean number of lesions depended upon the time of counting However, the differences observed among mouse strains were not consistent and were not statistically significant. Neither did the day of counting lesions (early versus later) make a statistically significant difference for any single mouse strain.

Tail lesions were counted in 3-4 week-old (19.5 mgs) and 10 week-old (34.6gms) CD-1 mice. Although slightly more tail lesions developed using younger mice, the differences were not statistically significant (p=0.28 by paired t-test).

Ara-A , AVS 1752, was more effective than ribavirin, AVS 1, in the vaccinia virus tail lesion model.

The parent drug, adenosine n'-oxide , 1985, and several analogues were tested at several drug dose levels. Drugs tested , in addition to 1985, included: 2911, 3607, 3679 and 4224. The order of efficacy, from most potent to least, was 4224, 1985, 3679, and 3607, and 2911 at 300 mg/kg. The analogue 4224 is also more potent than the parent and other analogues at lower drug dose levels (200 mg/kg).

A protocol has been developed for testing of an immunomodulator in the yellow fever virus primate model. Implementation of the protocol was delayed because of an potential intercurrent outbreak of Ebola virus in cynomolgous monkeys.

FOREWORD

In conducting the research described in this report, the investigator 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) 86-23, Revised 1985).

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INTRODUCTION

This report is divided into four sections. The results of drug testing in three different murine models are shown in the first three sections. The results of drug testing in a primate model are given in the fourth section.

SECTION ONE: REPORT OF LCMV DRUG TESTS

1.0 PREPARATION OF AN LCM VIRUS STOCK

We had been using a unique strain of LCM virus (LCMV) in drug tests in the LCMV model. The virus strain in use has a high peripheral pathogenicity for adult mice which is unusual for LCMV infection of mice. We derived this strain by passage in C3H/J mice. In this contract period, we had difficulties in propagating our LCMV stock. Problems were encountered because the virus stock used for our drug tests has been prepared in C3H/J mice. The C3H/J strain of mouse was not available to us because of a fire at Jackson laboratories.

Fortunately, bagged and frozen adult infected C3H/J mice had been held aside in the Revco freezer (-70) for such an emergency. Virus was harvested from the brains of these frozen mice and was titrated in CF1 mice.

Efforts were undertaken to prepare a stock of LCM virus (LCMV) for additional drug testing. First, we had to find a mouse strain which would support LCMV replication in such a way that peripheral virulence for random-bred mice was maintained. Virus isolated from the brains of C3H/J mice is lethal for random bred mice when inoculated peripherally (i.p.).

Two types of three-week old mice were obtained from Charles River Laboratories (Kingston). These included random-bred CF1 mice and C3H/HEN mice. LCMV from C3H/J mice was inoculated into both CF1 and C3H/HEN mice. Virus was harvested on the seventh day from the brains of these two types of infected mice. (The basis for choosing the 7th day was dictated by our previous experience using C3H/J mice in which virus titer was highest on day 7 after inoculation.) Virus stocks were prepared from virus both CF1 and C3H/HEN mice. After preparation, these virus stocks were titrated in parallel in CF1 mice (six-week old, random bred). The results are summarized in the Table 1.

1.1 MORTALITY WITH LCMV STOCKS AFTER PROPAGATION IN 3 DIFFERENT STRAINS OF MICE

The important parameter was how the mice died in these intraperitoneal (ip) titrations. In testing antiviral drug activity, it was important to find a dose which gave uniform mortality. The best virus stock was the CH3/HEN stock which gave uniform mortality over a range of virus dilutions. These data are shown in Table 2.

1.2 RESULTS OF LCMV DRUG TESTS

Ninety-three drugs were tested against LCMV in this adult mouse model. Fourteen drugs gave VR scores >1.2 which places these drugs in the top 90th percentile of all drugs tested in the LCMV model. The dates of the drug tests, the AVS numbers, VR scores, virus and drug doses used are given in the Tables 3-8

TABLE 1. SUMMARY OF LCM VIRUS TITERS AFTER PROPAGATION OF LCMV IN 3 DIFFERENT STRAINS OF MICE

Mouse strain for virus propagation	Virus titer in CF1 mice	Number of ampoules
C3H/HEN	$10^{-6.0}$/0.2 mls ip	20
C3H/J	$10^{-5.2}$/0.2 mls. ip	29
CF1	$10^{-5.4}$/0.2 mls. ip	8

TABLE 2. MORTALITY OF RANDOM BRED (CF1) MICE AFTER PROPAGATION OF LCMV IN 3 DIFFERENT STRAINS OF MICE

MOUSE STRAIN USED FOR VIRUS PROPAGATION:		
CH3/HEN STRAIN		
VIRUS DILUTION	*DEAD*	*ALIVE*
10-2	5	0
10-3	5	0
10-4	5	0
10-5	5	0
10-6	2	2
CF1 RANDOM-BRED		
VIRUS DILUTION	*DEAD*	*ALIVE*
10-2	4	1
10-3	5	0
10-4	4	1
10-5	3	2
10-6	3	2
C3H/J STRAIN (JAX)		
VIRUS DILUTION	*DEAD*	*ALIVE*
10-1.7	5	0
10-2.7	4	1
10-3.7	5	0
10-4.7	4	1
10-5.7	2	3
10-6.7	0	5

TABLE 3. RESULTS OF TESTING IN THE LCM MODEL 3/13/89

AVS NUMBER	VR SCORE	VIRUS DOSE LD_{50}'S	DRUG DOSE MG/KG	NO. OF DRUG DOSES
208	1.2	25	50	5
2870	0.9	25	50	5
2877	0.6	25	50	5
2878	0.6	25	50	5
2957	0.6	25	50	5
2958	0.8	25	50	5
4230	0.7	25	50	5
4605	0.8	25	50	5
4607	0.8	25	50	5
4608	0.8	25	50	5
4610	1.0	25	50	5
4611	1.8	25	50	5
4612	0.7	25	50	5
4614	0.7	25	50	5
5031	0.8	25	50	5

TABLE 4. RESULTS OF TESTING IN THE LCM MODEL 4/3/89

AVS NUMBER	VR SCORE	VIRUS DOSE LD_{50}'S	DRUG DOSE MG/KG	NO. OF DRUG DOSES
208	1.6	100	50	3
1988	0.9	100	50	3
4613	1.3	100	50	3
4614	1.0	100	50	3
4615	0.9	100	50	3
4618	1.1	100	50	3
4619	1.0	100	50	3
4620	0.9	100	50	3
4621	1.0	100	50	3
4622	1.3	100	50	3
4623	1.0	100	50	3
4624	1.1	100	50	3
4720	1.8	100	50	3
4721	1.1	100	50	3
4727	1.0	100	50	3
4737	0.9	100	50	3
4738	1.1	100	50	3
4739	0.9	100	50	3
4748	0.9	100	50	3
4750	0.9	100	50	3
4751	0.9	100	50	3
4758	1.1	100	50	3
4760	0.8	100	50	3
4761	0.9	100	50	3
4762	0.9	100	50	3

TABLE 5. RESULTS OF TESTING IN THE LCM MODEL 6/12/89

AVS NUMBER	VR SCORE	VIRUS DOSE LD_{50}'S	DRUG DOSE MG/KG	NO. OF DRUG DOSES
205	1.8	100	100	5
4782	1.1	100	75	5
4783	1.0	100	75	5
4784	1.0	100	75	5
4785	1.1	100	75	5
4786	1.1	100	75	5
4787	1.2	100	75	5
4789	1.1	100	75	5
4790	1.0	100	75	5
4791	1.0	100	75	5
4795	1.2	100	75	5
4796	1.5	100	75	5
4800	1.1	100	75	5
4801	1.0	100	75	5
4802	1.2	100	75	5
4803	1.0	100	75	5
4804	1.0	100	75	5
4805	1.0	100	75	5
4806	1.2	100	75	5
4807	1.2	100	75	5
4808	1.3	100	75	5
4809	1.0	100	75	5
4810	1.2	100	75	5
4811	1.0	100	75	5
4812	0.9	100	75	5
4793	TOXIC			
4798	TOXIC			

FOOTNOTE TO TABLES:

DESCRIPTION OF THE LCMV MODEL

Adult mice are inoculated with 50 mg/kg of drug i.p. in a volume of 0.4 mls to 0.7 mls depending upon the weight of the individual mice. Forty-five minutes later, mice are inoculated with 50 LD50's of LCM virus (LCMV) i.p. The virus strain is propagated by intracerebral passage in inbred C3H mice. Random bred CF-1 mice from Charles River are used for drug tests. The identity of the virus strain has been monitored by examination of infected mouse tissue by immunofluorescence. The virus stock titers 5.0 to 5.4 log LD50's by intraperitoneal inoculation of random bred mice. The drug diluent was DMEM except where specific otherwise by USAMRIID data sheets.

CORRELATION BETWEEN THE GEOMETRIC MEAN SURVIVAL TIME OF LCMV INFECTED CONTROL MICE (VC) AND VIRUS DOSE.

The relationship between the VC and the virus dose in the LCMV model was presented in detail in the previous year's report.

TABLE 6. RESULTS OF TESTING IN THE LCM MODEL 6/19/89

AVS NUMBER	VR SCORE	VIRUS DOSE LD_{50}'S	DRUG DOSE MG/KG	NO. OF DRUG DOSES
208	1.2	200	100	3
4817	0.9	200	77	5
4818	1.1	200	77	5
4820	1.0	200	77	5
4821	1.0	200	77	5
4822	1.1	200	77	5
4823	1.1	200	77	5
4824	1.0	200	77	5
4825	1.0	200	77	5
4826	1.2	200	77	5
4828	1.0	200	77	5
4829	1.0	200	77	5
4830	1.0	200	77	5
4831	1.3	200	77	5
4833	0.9	200	77	5
4834	1.1	200	77	5
4835	1.0	200	77	5
4836	1.0	200	77	5
4840	1.0	200	77	5
4841	1.0	200	77	5
4814	TOXIC			
4815	TOXIC			
4816	TOXIC			
4837	TOXIC			
4838	TOXIC			
4839	TOXIC			

FOOTNOTE TO TABLES:

DESCRIPTION OF THE LCMV MODEL

Adult mice are inoculated with 50 mg/kg of drug i.p. in a volume of 0.4 mls to 0.7 mls depending upon the weight of the individual mice. Forty-five minutes later, mice are inoculated with 50 LD50's of LCM virus (LCMV) i.p. The virus strain is propagated by intracerebral passage in inbred C3H mice. Random bred CF-1 mice from Charles River are used for drug tests. The identity of the virus strain has been monitored by examination of infected mouse tissue by immunofluorescence. The virus stock titers 5.0 to 5.4 log LD50's by intraperitoneal inoculation of random bred mice.

CORRELATION BETWEEN THE GEOMETRIC MEAN SURVIVAL TIME OF LCMV INFECTED CONTROL MICE (VC) AND VIRUS DOSE.

The relationship between the VC and the virus dose in the LCMV model was presented in detail in the previous year's (1989) report.

SECTION TWO: REPORT OF CCHF VIRUS STUDIES

2.0 ISOLATION OF RIBAVIRIN-RESISTANT VIRUS FROM RIBAVIRIN-TREATED ANIMALS

Earlier we have reported that mice infected with 2.5 LD50's of CCHF virus from liver tissue of CCHF infected and drug-treated mice were not protected by ribavirin treatment. The VR score in mice treated with 50 mg/kg was 1.0 and the VR score in mice treated with 100 mg/kg was 0.9. The ribavirin VR scores in mice infected with 2.5 LD50's of CCHF liver virus which had not been passaged through ribavirin-treated animals were 2.7 and 3.0 for 50 mg/kg and 100 mg/kg respectively.

2.1 IDENTITY OF RIBAVIRIN-RESISTANT VIRUS

The identity of the ribavirin resistant virus has been established by mouse neutralization test using acetone-ether extracted CCHF virus mouse polyclonal antibody. There were no breakthrough mice in the neutralization test.

2.2 STABILITY OF RIBAVIRIN RESISTANT VIRUS

Repeated passage (3 passages) of the virus through ribavirin-treated animals resulted in a progressive loss of virus virulence after ip inoculation of infant mice. After three passages, there was no mortality in untreated animals. This result suggested that the virus titer in the liver tissue of resistant mice was decreasing with each passage. We have not looked at the virus titer in other organs during the course of infection with ribavirin-resistant virus. It may be that the pathogenesis of ribavirin resistance virus is different from that of the parent virus.

2.3 ATTEMPTS TO ISOLATE RIBAVIRIN RESISTANT VIRUS FROM UNTREATED MICE AND TO RE-ISOLATE RESISTANT VIRUS FROM TREATED MICE.

There were two questions that we felt should be answered. First, we wanted to know if ribavirin-resistant virus could be isolated from untreated mice. Second, we wanted to know if we could duplicate the experiment described above. We particularly needed to duplicate our previous experiment because we needed a larger resistant virus stock. To accomplish these goals, we harvested CCHF virus from liver tissue of untreated and ribavirin-treated mice at various times after infection. In our initial experiment, we isolated virus on the seventh day from mice with no clinical signs (NCS). Therefore, we followed the same general protocol in these experiments. The protocol followed is summarized in Table 7.

2.4 RIBAVIRIN RESISTANCE AND MORTALITY

Examination of mortality alone suggested that ribavirin-resistant virus could be isolated from both untreated animals and treated animals. After isolation of virus from both untreated and treated groups, there were instances where a significant number of animals died despite ribavirin treatment. (Table 8A).

2.5 RIBAVIRIN-RESISTANCE AND THE GEOMETRIC MEAN SURVIVAL TIME (VR SCORE).

Although mice died after treatment with ribavirin, the geometric mean survival time was almost always significantly elevated (1.2 or greater). This occurred in every case where the virus source was from untreated mice or mice treated with ribavirin at 50 mg/kg. If resistance is measured by a change in both mortality and VR score, then we have 3 substrains, derived from the same parent, which are ribavirin resistant. The parent was derived by passage through mice treated with 100 mg/kg of ribavirin, as was true in the initial experiment. These data are shown in Table 8B.

TABLE 7. SUMMARY OF RIBAVIRIN RESISTANCE STUDIES IN THE CCHF MOUSE MODEL

MOUSE GROUP NUMBER	DRUG TREAT-MENT	VIRUS DILUTION	DATE OF HARVEST	SICK OR NCS	DATE OF PASSAGE	VENT NO
2904	DMEM	-4	4/29/89	S	5/17	3013,3014,3015
2905	DMEM	-5	4/29	?	5/12	2989,2990,2991
2905	DMEM	-5	5/1	W	6/2	3059,3060,3061
2905	DMEM	-5	5/1	S	5/12	2992,2993,2994
2907	50 mg AVS 1	-2	5/1	S	5/17	3016,3017,3018
2908	50 mg AVS 1	-3	5/1	S	6/2	3062,3063,3064
2909	50 mg AVS 1	-4	4/29	?	5/12	2998,2999,3000
2910	50 mg AVS 1	-5	5/1	W	5/12	3001,3002,3003
2910	50 mg AVS 1	-5	4/28	S	5/12	2995,2996,2997
2910	50 mg AVS 1	-5	5/1	?	6/2	3056,3057,3058
2911	50 mg AVS 1	-6	5/2	?	5/15	3004,3005,3006
2912	100 mgAVS 1	-2	4/29	W	5/31	2980,2981,2982
2912	100 mgAVS 1	-2	4/28	W	5/31	3022,3023,3024
2913	100 mgAVS 1	-3	4/28	W	5/15	3007,3008,3009
2913	100 mgAVS 1	-3	5/1	?	5/31	3025,3026,3027
2913	100 mgAVS 1	-3	5/2	?	5/31	3019,3020,3021
2914	100 mgAVS 1	-4	5/4	W	5/15	3010,3011,3012
2914	100 mgAVS 1	-4	5/4	S	5/11	2983,2984,2985
2992	50 mgAVS 1	-1	5/2o	W	5/18	2986,2987,2988
3014	50 mgAVS 1	-1	5/23,5/24	3S	6/30	3168,3169,3170
3016	100 mgAVS 1	-1	5/25	2W	6/30	3171,3172,3173
3108	50 mgAVS 1	-1	6/22	2W	6/30	3174,3175,3176
3111	50 mgAVS 1	-1	6/23	2S	6/30	3177,3178,3179
2990	100 mgAVS 1	-1	5/21,5/23	W	6/15	3180,3181,3182
3021	100 mgAVS 1	-1	6/7	2W	6/15	3104,3105,3106
3023	50 mgAVS 1	-1	6/9	?,S	5/31	3107,3108,3109
						3110,3111,3112

TABLE 8A. RIBAVIRIN RESISTANCE IN THE CCHF MODEL: MORTALITY

DRUG TREATMENT	VIRUS DILUTION	DAY OF HARVEST	RESULTS OF PASSAGE (MORTALITY)		
			DMEM	50 MG/KG	100 MG/KG
0	-4	8 (SICK)	8/8	8/8	3/8
0	-5	9 (SICK)	8/8	5/8	5/7
0	-5	10 (NCS)	0/8	0/8	0/8
0	-5	11 (SICK)	7/8	0/8	0/8
50	-1	6 (SICK)	7/8	3/8	0/8
50	-1	7 (NCS)	6/8	3/8	1/8
50	-1	9 (SICK)	8/8	8/8	0/8
50	-1	7 (NCS)	0/8	0/8	0/8
50	-1	9 (NCS)	0/8	0/8	0/8
50	-2	10 (SICK)	4/8	0/8	0/8
50	-3	10 (SICK)	7/8	1/8	0/8
50	-4	8 (SICK)	6/8	ND	0/8
50	-4	9 (NCS)	0/8	0/8	0/8
50	-5	7 (SICK)	2/8	2/8	2/8
50	-5	10 (SICK)	0/8	0/8	0/8
50	-5	11 (SICK)	0/8	0/8	0/8
50	-6	8 (NCS)	0/8	0/8	0/8
100	-1	7 (NCS)	4/8	1/8	0/8
100	-1	8 &11 (SICK)	1/8	0/8	0/8
100	-1	8 (NCS)	4/8	0/8	0/8
100	-1	9 (NCS)	0/8	0/8	0/8
100	-2	7 (NCS)	8/8	5/8	4/8
100	-3	7 (NCS)	3/8	3/8	1/8
100	-3	10 (SICK)	8/8	8/8	5/8
100	-3	11 (NCS)	0/8	0/8	0/8
100	-4	13 (SICK)	1/8	0/8	0/8
100	-4	13 (NCS)	0/8	0/8	0/8

Footnote:

Ribavirin-resistance in the CCHF model. Two ribavirin-treated (single dose, 100 mg/kg) mice with no clinical signs of illness were necropsied on the seventh day after infection. Virus was isolated from pooled liver and pooled blood by ip passage in other infant mice. The infectious virus titer in the liver (4.0 $\log_{10}$ LD_{50}'s/gram of tissue) was higher than the titer in the blood (2.3 $\log_{10}$ LD_{50}'s/ml).

The original virus from liver tissue from ribavirin treated mice was used as source in a ribavirin drug test. Mice were infected with a low dose of the liver virus (25 LD_{50}'s) and treated with ribavirin at either 50 mg/kg or 100 mg/kg. The VR in mice treated with 50 mg/kg was 1.0 and the VR in mice treated with 100 mg/kg was 0.9. The ribavirin VR's in mice infected with 25 LD_{50}'s of CCHF liver virus which had not been passaged through ribavirin-treated animals were 2.7 and 3.0 for 50 mg/kg and 100 mg/kg respectively. NCS means no clinical signs.

TABLE 6B. RIBAVIRIN RESISTANCE IN THE CCHF MODEL: MORTALITY AND GEOMETRIC MEAN SURVIVAL TIME

VIRUS SOURCE	TREATMENT OF RECIPIENT MICE					
	DMEM		50 mg/kg		100 mg/kg	
	Mortality	VC Score	Mortality	VR Score	Mortality	VR Score
(1) DMEM, 10-4, D8, SICK	8/8	8.0	7/8	1.2	3/8	2.5
(2) DMEM, 10-5, D9, SICK	8/8	7.6	5/8	1.8	5/7	1.8
(3) DMEM, 10-5, D10 (NCS)	0/8	NA	0/8	NA	0/8	NA
(4) DMEM, 10-5, D11, SICK	7/8	7.8	0/8	3.5	0/8	3.5
(5) AVS1, 50 MG/KG, 10-1, D9, SICK	8/8	9.0	8/8 ***	1.2	0/8	3.2
(5a) ***AVS1, 50/MG/KG, D7, SICK	8/8	12.6	8/8****	1.2	8/8	1.3
(5b) ****AVS1, 50 MG/KG, D7, SICK	6/8	12.6	3/8	1.5	1/8	2.0
(6) AVS1, 50 MG/KG, 10-2, D10, SICK,	4/8	16.9	0/8	1.7	0/8	1.7
(7) AVS1, 50 MG/KG, 10-5, D7, SICK	2/8	21	2/8	1.2	2/8	1.0
(7) AVS1, 100 MG/KG, 10-2, D7 (NCS)	8/8	9.2	5/8	1.5	4/8	1.6
(8) AVS1, 100 MG/KG, 10-3, D7, NCS	3/8	19.5	3/8 *	0.9	1/8 **	1.2
(8a) *AVS1, 50 MG/KG, D8, SICK	8/8	8.1	8/8	1.0	7/8	1.2
(8b) **AVS1, 100 MG/KG, D7, SICK	4/8	15.4	4/8	0.9	4/8	1.0

* INDICATES A MOUSE PASSAGE;
THE VIRUS TITER (LD50) IN DMEM IS 10-5.3 (0.02 ml., IP)

Footnote

Attempts to isolate ribavirin resistant virus from placebo-treated mice and to re-isolate resistant virus from ribavirin-treated mice. CCHF virus substrains were tested for ribavirin-sensitivity by harvesting virus from liver tissue of placebo-treated and ribavirin-treated mice at different times after infection with varying virus doses. Representative results are shown in detail in table 6b. A parent virus was derived by passage through mice treated with 100 mg/kg of ribavirin, as in the first experiment described above. Substrains, derived from this parent, were resistant (VR ≤ 1.0) to treatment with 50 mg/kg of drug, in each case, sensitivity to ribavirin at 100 mg/kg was also reduced. NCS means no clinical signs

SECTION THREE: SUMMARY OF STUDIES WITH VACCINIA VIRUS

3.0 ORIGIN AND PASSAGE OF THE COPENHAGEN STRAIN OF VACCINIA VIRUS .

Initially, one vial of two strains of ectromelia virus virus (IHD strain and the Copenhagen strain) were received at Yale. These materials had been thawed before they were shipped to us on dry ice. The IHD strain was received as an egg membrane suspension and the Copenhagen strain was received as a calf lymph. The actual designation of the virus strains we received were Emory and Koppe. We assumed that the Emory strain is the IHD strain and the Koppe strain is the Copenhagen strain although we could be sure of the accuracy of this assumption. The virus strains were passaged in VERO cells.

The Koppe strain contained material which was instantly toxic to VERO cells. The original material at a 1:100 dilution was put through a 0.22 mμ filter and was then again inoculated into Vero cells . Eighteen hours later, the cells were examined by IFA using an ectromelia virus antibody. The cells were IFA positive. These cultures were used to make a tissue culture stock. This stock was passaged into suckling mice because the tissue culture grown virus did not produce tail lesions in mice. An infant mouse brain stock was prepared with a titer of $10^{3 \cdot 5}$/0.02 mls in infant mice. The Emory strain was passed in Vero cells. CPE was found 18 hours later. The cultures were harvested and a stock was prepared. This virus was passed into infant mice and a stock was made from brain tissue. The stock was designated as Copenhagen.

3.1 TAIL LESIONS WITH THE COPENHAGEN STRAIN OF VACCINIA.

The methodology followed here was adapted from a protocol developed by Boyle et al..[1] The two strains were inoculated into tail veins of six-week old male random-bred mice. Mice developed lesions on the eighth day both at 1:10 and 1:100 dilutions of the Koppe virus strain stock. Following inoculation of the Emory strain, lesions developed on the eight day at 1:10, but not at the 1:100 dilution. Lesions could not be observed after the 14 days. There was no mortality in any group inoculated.

Lesions could be easily quantitated by staining tails with a mixture of methylene blue and fluorescein. The Koppe strain produced the more severe lesions. Therefore, this strain was used in our initial tests to standardize the model. Photographs of lesions are shown in Figures 1 and 2.

Subsequently, we received from Dr. Joel Dalrymple four different virus strains. A limited description of each follows.

3.2 OTHER VACCINIA VIRUS STRAINS TESTED FOR PRODUCTION OF TAIL LESIONS.

3.2.1 Copenhagen VAX 3H1 Master Oct. 86.

The Copenhagen strain of virus was originally sent to USAMRIID by Enzo Paoletti with very little passage history other than this was the virus that apparently served as the parent for the recombinant rabies vaccinia vaccine. At USAMRIID, the was a plaque cloned by a procedure involving filtration and sonication. The 3

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[1] Evaluation of antiviral compounds by suppression of tail lesions in vaccinia-infected mice. Boyle, JJ, Kaff, RI, Stewart, R.C. (1966) Antimicrobial Agents and Chemotherapy, 536-539)

designation means 3 times plaque cloned and the H line is one of 10 separate plaques that were carried through the process and the final 1 in the designation means that it was the first of duplicate plaques picked on the final passage. This particular plaque clone was selected quite arbitrarily but was among the highest titered of the plaques tested at USAMRIID. It was also shown to be free of what was originally though to be variable termini on a restriction fragment which suggested the virus was indeed plaque cloned. At Yale, this strain was designated as 3PP.

3.2.2 Lister VAX 3D1 Master Oct 86

This virus was derived from the Connaught strain of vaccinia. It is the master seed for Connaught's smallpox vaccine which was purchased by Col. Barquist a few years ago and was originally derived from the New York Board of Health Strain. This virus was plaque cloned at USAMRIID as described above and was grown to master seed levels (3MRC-5) at the Salk Institute to serve as a master seed for a new and improved smallpox vaccine that USAMRIID is planning to make. It will also serve as the parent for USAMRIID's recombinant vaccines. It is designated white plaque to different it from the virus described below. At Yale, this strain was designated as Lister.

3.2.3 Vaccinia virus Plasmid 9.1 11K P Conn 3E1 Blue #62 Lac Z pn7 2MRC5

This virus is identical to the one described above (Conn 3E1) but USAMRIID inserted the Lac Z gene coding for beta-galactosidase into the thymidine kinase gene. They used a plasmid called 9.1 and the beta-gal gene is under the control of the vaccinia 11 K promoter. It was plaque cloned 7 times in the selection and went through 2 MRC5 passes to build the seed. It was thought to be of reduced virulence because of the absence of a functional TK gene. At Yale, this strain was designated as Plasmid.

3.2.4 VAX W.T. Day 3 MRC5 Sept 87

This is an old lab strain known as WR and is called at USAMRIID W.T. for wild type. It was obtained from Shu Lok Hu when he was at Molecular Genetics, Inc. and there is no passage history other than it has a history of passage in mouse brain tissue to select for mouse neurovirulence. USAMRIID has shown it to be more mouse neurovirulent than any other strain of vaccinia virus which has been handled there. At Yale, this strain was designated as Wild Type.

3.3 VACCINIA VIRUS TAIL LESIONS AND VACCINIA VIRUS STRAINS.

Each of 5 virus strains was grown in Vero cells and titrated by plaque formation. The lowest plaque titer was obtained with the wild type (398 PFU/0.1 mls), the highest with the Copenhagen strain (630,957 PFU/0.1 mls). The plasmid and 3PP strains had identical plaque titers, 25,199 PFU/0.1 mls. Mice were inoculated with the lowest possible dose, 398 PFU of each strain. Lesions were counted. The wild type was the most virulent strain requiring only 9 PFU to produce a lesion. The Lister, 3PP, and Copenhagen were the next more virulent in that order with 66, 284, and 995 PFU, respectively, required to produce lesions. The plasmid strain did not produce lesions with this inoculum dose (Table 9A).

The experiment was repeated using the maximum number of PFUs for each virus as inoculum. The results were generally similar. The most virulent virus strain was the wild type which required only 9 PFU to produce a tail lesion. The Lister, Copenhagen and Plasmid strains were the next most virulent in that order requiring 249, 7,336, and 17,942 PFU, respectively, to produce a single lesion. Using a high virus dose inoculum, the 3PP strain required 62,797 PFU to produce a lesion (Table 9B).

On the basis of these experiments, a decision was made to conduct future tests with the Copenhagen strain since it was of intermediate virulence in the mouse tail lesion model.

3.4 CORRELATION BETWEEN VIRUS DOSE AND TAIL LESIONS.

One of the first needs was to establish whether there a linear relationship existed between virus dose and the number of tail lesions in vaccinia virus infected mice. In addition, we wanted to determine the relationship between mouse virulence and lesion formation. A stock of the Copenhage strain of vaccinia was prepared and titrated in infant mice. The LD_{50} titer in infant mice was over 2 logs lower than the PFU titer. Dilutions of this Copenhagen virus stock was inoculated into groups of twenty mice for each dilution. The correlation (r^2) between lesions in DMEM-treated mice and LD_{50} virus dose was 0.9 (Figure 3A). A second titration was done using the PFU titer as a basis for virus dilutions, the data showed that 3700 PFUs were required to produce a single tail lesion. The correlation coefficient was 0.8 (Figure 3B). This is in virtual agreement with the observations from the initial set of experiments reported above. Passage in infant mouse tissue had not altered this property of the Copenhagen virus stock.

These data also show that the virus dose range with the least variation was that dose which produced between 16 and 25 lesions per mouse. That format for drug studies was adopted using a minimum of 20 mice per group. The number of mice used per group was dependent upon the amount of drug which could be provided. In some cases, that amount was highly variable. In no case were fewer than 20 mice used.

3.5 CORRELATION BETWEEN VACCINIA VIRUS TAIL LESIONS AND MOUSE STRAIN.

Two inbred mouse strains, BALB/C and NU/NU, and two random bred mouse strains CF-1 and CD-1 were inoculated with the Copenhagen strain of vaccinia virus. Lesions were counted immediately after formation (days 6-7) and later just before the lesions were resolved (days 8-10) depending upon the individual mouse strain. The variation about the mean number of lesions depended upon the time of counting (Figures 4A and 4B). However, the differences observed among mouse strains were not consistent and were not statistically significant (Tables 10). Neither did the day of counting lesions (early versus later) make a statistically significant difference for any single mouse strain, although the data for the CD1 strain might be worth exploring further. (Table 10). T

In the protocol for the drug studies, the decision was made to count lesions daily until the lesions resolved and to compare results between controls and drug-treated animals on a daily basis.

3.6 SCREENING FOR A POSITIVE CONTROL DRUG.

Several variables had been resolved. We could produce lesions in a variety of mouse strains; a virus strain of intermediate virus virulence was chosen for screening. The major unresolved question was whether the tail lesions could be inhibited by drug treatment. The first drug screened was ribavirin. It was not effective in inhibiting tail lesions at doses under 250 mg/kg. However, it was significantly effective at 250 mg/kg (Figure 5 & Table 11).

A second drug, ara A (coded as AVS 1752), was also screened . It was more effective than ribavirin (AVS 1) in that less drug (mg/kg) was required to product a significant reduction in the number of tail lesions (Figure 6 & Table 12).

3.7 RESULTS OF DRUG TESTS IN THE VACCINIA VIRUS TAIL LESION MODEL.

Drugs were screened using a starting drug dose of 200 mg/kg and varying virus doses within the range described above. The effective drugs would then be retested using varying doses of drug. That was the research protocol.

The results of our first two drug tests are given in Tables 13 and 14. VR scores over 1.0 were observed in some cases where the differences in number of lesions between drug-treated and placebo-treated mice were not statistically significant by paired t-test. One reason for the discrepancy lies in the variation about the mean number of lesions. When variation about the means is large, it diminishes the significance of the difference between mean values in drug-treated and placebo-treated animals. Such variation is inherent in this test system in our hands and in the hands of others. For this reason, both the VR score and the statistical test (t-test) results are given.

The data with AVS 1752 showed that drug efficacy varied directly with both drug dosage and virus dose, as determined by the mean number of control lesions. The greater the drug dose, the higher the VR score. At any given drug dose, the lower the virus dose, the higher the VR score (Table 13).

3.8 CORRELATION BETWEEN TAIL LESIONS AND MOUSE WEIGHT & AGE.

One drug , AVS 3679, received special attention because our test results at 200 mg/kg did not demonstrate drug activity (Table 14). Several variables were posed which might account for a discrepancy between our test results and those of others. Among them was weight and age of the mouse and drug dose. Mouse weight and age had not been explored in our prior tests so we examined that variable as well as testing at a higher dose.

Tail lesions were counted in 3-4 week-old (19.5 mgs) and 10 week-old (34.6gms) CD-1 mice (Table 15). Although more tail lesions developed using younger mice, the differences were not statistically significant (p=0.28 by paired t-test). While weight made little difference in the outcome of the drug tests with AVS 3679, the drug dosage was critical. At 300 mg/kg, there was a significant reduction in the number of tail lesions as determined by both VR scores and t-test. (Table 15)

3.9 COMPARISON OF EFFICACY AMONG ADENOSINE N-OXIDES.

The parent drug, 1985, adenosine n\-oxide and several analogues were also retested at 300 mg/kg.: 2911, 3607, 3679 and 4224. The order of efficacy, from most potent to least, was 4224, 1985, 3679, and 3607, and 2911 at 300 mg/kg (Table 16). The analogue 4224 is also more potent than the parent and other analogues at lower drug dose levels (200 mg/kg) as shown previously in Tables 13 and 14. The correlation with tissue culture results as we have received them are as follows:

AVS #	VR range in tissue culture (range given in documents received at Yale)	VR in tail lesion model (range for all doses of drug and virus)
1985	3.4->7.2	0.98-2.26
2911	>1.9->2.1	1.07-1.6
3607	>2.4,>2.7	1.21-1.47
3679	2.8-3.3	0.80-1.96
4224	>2.6-2.8	2.52-3.55
COMPOUNDS OTHER THAN SECRIST 1985 ANALOGUES		
1752 (ARA A)	0.8-1.4	0.86-3.70
001 (RIBAVIRIN)	no information received	0.70-2.04

FIGURE LEGENDS

FIGURE 1. STAINED VACCINIA TAIL LESIONS (TOP)

Photograph of lesions after staining the tail of a random bred white mouse with a mixture of fluorescein and methylene blue. The mouse had been given vaccinia virus (Copenhagen strain) eight days earlier.

FIGURE 2. STAINED VACCINIA TAIL LESIONS (BOTTOM)

Photograph of a different mouse from the same inoculated groupbearing lesions after staining the tail of the mouse as described above. This mouse was inoculated with the same dose of the same strain of virus . The photograph was made on the eight day after inoculation and has been presented to show the usualvariation in lesion count among infected mice.

FIGURES

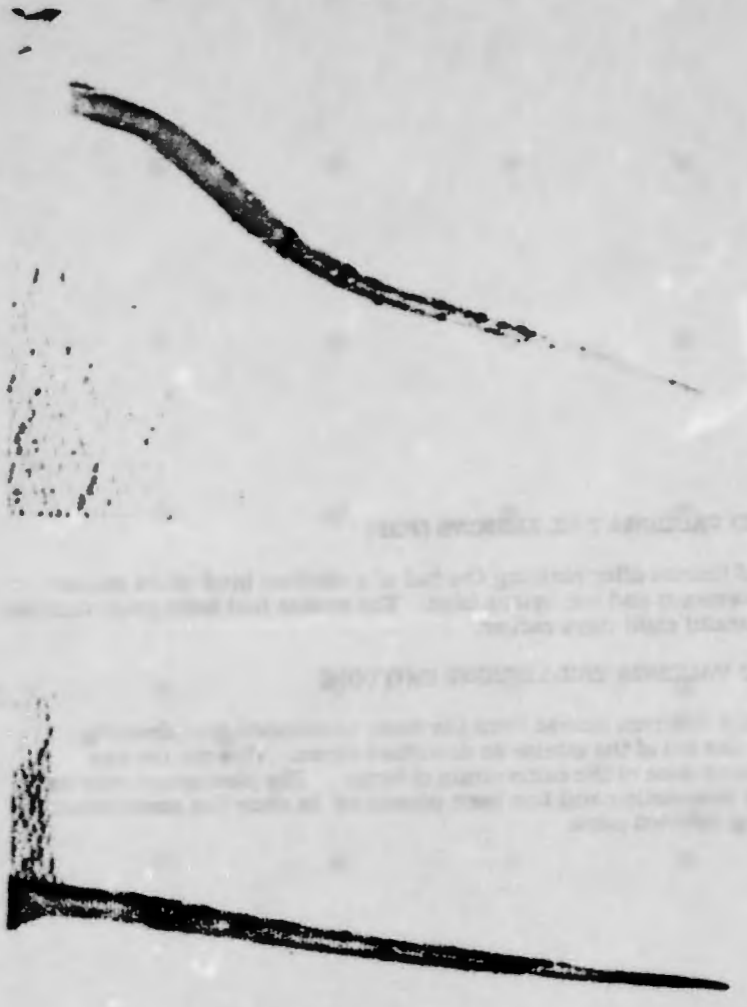


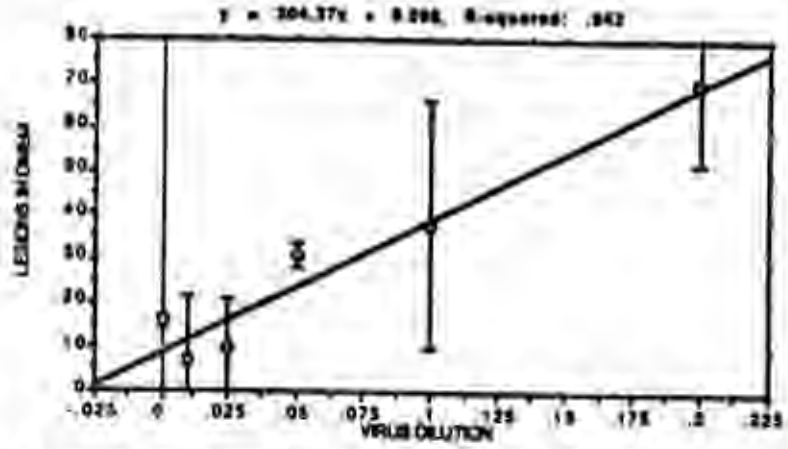
TABLE 9A. VACCINIA STRAINS AND TAIL LESIONS

VIRUS STRAIN	VIRUS TITER (PFU'S/0.1)	PFU'S INOCULATED	LESIONS (MEAN)	PFU/LESION
WILD TYPE	398	398	46	9
LISTER	3,981	398	6	66
3PP	25,119	398	1.4	284
COPENHAGEN	630,957	398	0.4	995
PLASMID	25,119	398	0.0	∞

TABLE 9B. VACCINIA STRAINS AND TAIL LESIONS

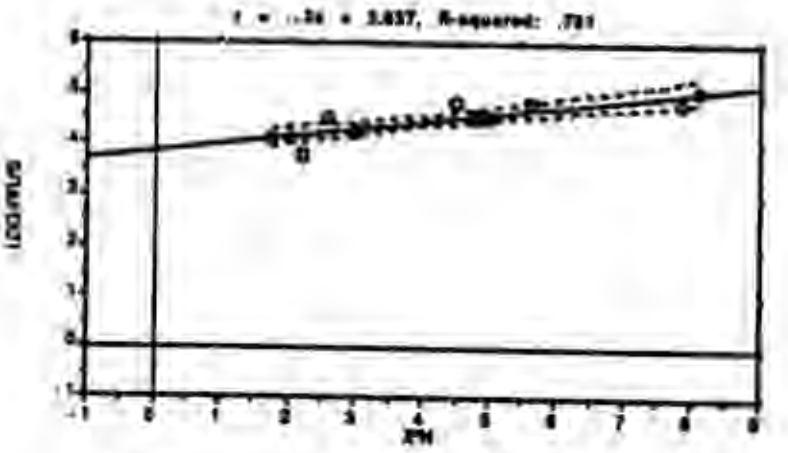
VIRUS STRAIN	VIRUS TITER (PFU'S/0.1)	PFU'S INOCULATED	LESIONS (MEAN)	PFU/LESION
WILD TYPE	398	398	46	9
LISTER	3,981	3,981	16	249
COPENHAGEN	630,957	630,957	86	7,336
PLASMID	25,119	25,119	1.4	17,942
3PP	25,119	25,119	0.4	62,797

FIGURE 3A. CORRELATION BETWEEN VIRUS DOSE AND TAIL LESIONS IN CF1 MICE



The titer of the Koppe virus stock used in this experiment was $10^{3.7}$ LD_{50} in infant mice [6]. Therefore, a dilution of 1/5 (0.20) is equivalent to a virus dose of 1000 LD_{50}; a dilution of 0.10 (1/10) is equivalent to 500 LD_{50}; 1/20 (0.05) = 250 LD_{50}; 1/40 (0.025) = 125 LD_{50}; 1/100 (0.01) = 50 LD_{50}; 1/1000 (0.001) = 5 LD_{50}.

FIGURE 3B. CORRELATION BETWEEN TRANSFORMED LESION COUNT AND VACCINIA VIRUS DOSE†



†The virus strain used is Copenhagen. The PFU titer was determined in Vero cells. Note that over 3700 PFU's are required with the Copenhagen strain before a single tail lesion is observed. This is consistent with earlier data which showed that between 1000 and 7000 PFU'S were required to produce a single tail lesion with the Copenhagen strain of Vaccinia.

FIGURE 4A. EFFECT OF TIME AND MOUSE STRAIN ON DEVELOPMENT ON TAIL LESIONS IN VACCINIA VIRUS INFECTED MICE: EARLY LESION COUNTS

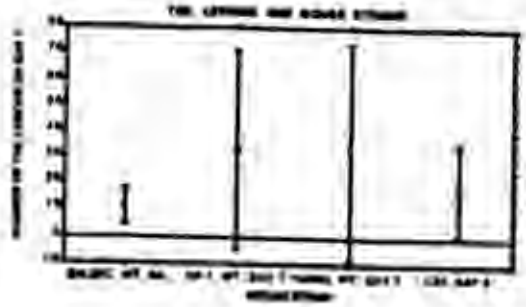


FIGURE 4B. EFFECT OF TIME AND MOUSE STRAIN ON DEVELOPMENT ON TAIL LESIONS IN VACCINIA VIRUS INFECTED MICE: LATE LESION COUNTS

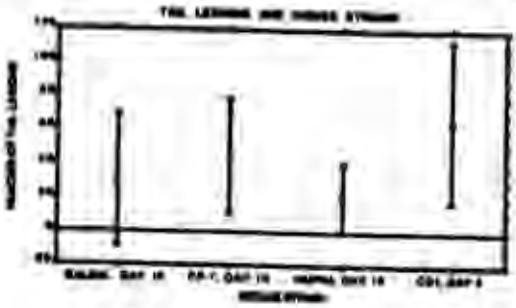
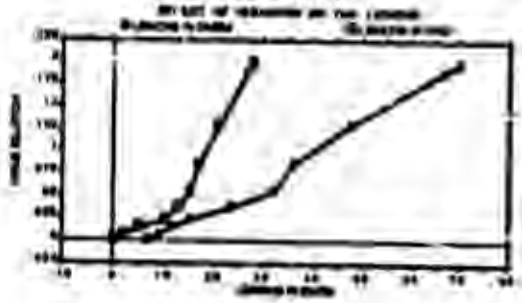


TABLE 10. STATISTICAL SIGNIFICANCE OF DIFFERENCES IN TAIL LESION COUNTS IN SELECTED MOUSE STRAINS INFECTED WITH VACCINIA VIRUS

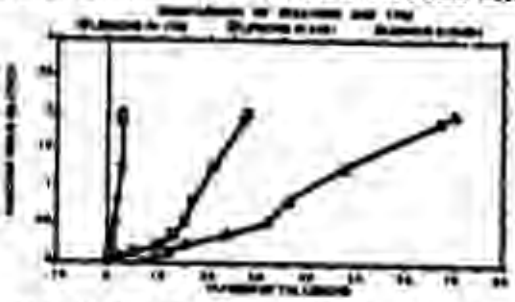
TAIL LESIONS AFTER VACCINIA VIRUS (KOPPE STRAIN) INFECTION OF SELECTED MOUSE STRAINS Paired t-Test Probabilities (2-tail)				
Mouse Strain	Mouse strain:			
	BALB/C DAY 10	CF-1 DAY 10	NU/NU DAY 10	CD-1 DAY 10
BALB/C DAY 7	0.30	0.12	0.37	0.07
CF-1 DAY 7	0.90	0.17	0.62	0.21
NU/NU DAY 7	0.68	0.70	0.39	0.43
CD1 DAY 4	0.66	0.18	0.92	0.06

FIGURE 5. EFFECT OF RIBAVIRIN ON TAIL LESIONS AFTER VACCINIA VIRUS INFECTION.



The titer of the Koppe virus stock used in this experiment was $10^{3.7}$ LD_{50} in infant mice (sc). Therefore, a dilution of 1/5 (0.20) is equivalent to a virus dose of 1000 LD_{50}; a dilution of 0.10 (1/10) is equivalent to 500 LD_{50}; 1/20 (0.05) = 250 LD_{50}; 1/40 (0.025) = 125 LD_{50}; 1/100 (0.01) = 50 LD_{50}; 1/1000 (0.001) = 5 LD_{50}.

FIGURE 6. COMPARISON OF RIBAVIRIN AND AVS 1752 ON DEVELOPMENT OF TAIL LESIONS IN VACCINIA VIRUS INFECTED MICE.



The titer of the Koppe virus stock used in this experiment was $10^{3.7}$ LD_{50} in infant mice (sc). Therefore, a dilution of 1/5 (0.20) is equivalent to a virus dose of 1000 LD_{50}; a dilution of 0.10 (1/10) is equivalent to 500 LD_{50}; 1/20 (0.05) = 250 LD_{50}; 1/40 (0.025) = 125 LD_{50}; 1/100 (0.01) = 50 LD_{50}; 1/1000 (0.001) = 5 LD_{50}.

TABLE 11. EFFECT OF RIBAVIRIN (250 MG/KG) ON TAIL LESIONS IN VACCINIA VIRUS (KOPPE STRAIN) INFECTED MICE: STATISTICAL ANALYSIS'

VIRUS DOSE (LD50'S)	PAIRED T VALUE	PROB. (2-TAIL)
1000	22.198	0.0003
500	1.705	0.1163
250	3.246	0.0176

TABLE 12. EFFECT OF AVS 1752 ON TAIL LESIONS IN VACCINIA VIRUS (KOPPE STRAIN, 500 LD50'S) INFECTED MICE: STATISTICAL ANALYSIS

DRUG CONCENTRATION	T VALUE	PROBABILITY
200 MG/KG	2.185	0.05
100 MG/KG	3.047	0.02
50 MG/KG	-0.424	0.34

TABLE 13. DRUG EFFICACY REPORT: VACCINIA VIRUS IN THE TAIL LESION
MODEL: 1/23/30

AVS #	DRUG DOSE † (MG/KG)	CONTROL MEAN LESIONS‡ $\sqrt{x^a}$	DRUG MEAN LESIONS‡ $\sqrt{x^a}$	VR CONTROL ÷ DRUG	P (T-TEST PAIRED ONE-TAIL)
1752	200	4.504	1.561	**2.85**	0.06
1752	100	4.504	1.216	**3.70**	**0.02**
1752	50	4.504	5.247	**0.86**	0.34
1752	200	2.578	0.559	**4.61**	**0.03**
1752	100	2.578	1.666	**1.55**	0.34
1752	50	2.578	2.128	**1.21**	0.48
0001	250	8.142	3.982	**2.04**	**0.0001**
0001	250	5.622	4.416	**1.27**	0.06
0001	250	2.994	3.175	**0.94**	0.42
0001	250	2.215	3.162	**0.70**	0.17
2911	200	7.841	4.829	**1.60**	0.08
2911	200	1.746	1.629	**1.07**	0.45
4224	200	7.841	3.115	**2.52**	**0.04**
4224	200	1.746	0.683	**2.56**	0.07

†Five doses of drug were given, beginning two days before virus inoculation and continuing for three day after virus inoculation. Lesions were counted on day 10. Drugs were given ip.

‡Where the mean number of lesions is equal to the average of $\sqrt{x^a}$ where x^a is the number of lesion per mouse. [EVALUATION OF ANTIVIRAL COMPOUNDS BY SUPPRESSION OF TAIL LESIONS IN VACCINIA-INFECTED MICE. BOYLE, JJ, HAFF, R.L, STEWART, R.C., (1966) ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, P536-539] The virus rating (VR) is a ratio of the control over drug-treated.

TABLE 14. DRUG EFFICACY REPORT: VACCINIA VIRUS IN THE TAIL LESION MODEL: 1/24/90

AVS #	DRUG DOSE † (MG/KG)	CONTROL MEAN LESIONS‡ $\sqrt{x^a}$	DRUG MEAN LESIONS‡ $\sqrt{x^a}$	VR CONTROL ÷ DRUG	P (T-TEST PAIRED ONE-TAIL)
1985	200	4.84	4.92	0.98	0.48
1985	200	4.56	2.96	1.54	0.17
3607	200	4.84	4.01	1.21	0.35
3607	200	4.56	3.10	1.47	0.21
3679	200	3.62	3.90	0.93	0.35
3679	200	1.77	2.94	0.60	0.06

†Five doses of drug were given, beginning two days before virus inoculation and continuing for three day after virus inoculation Lesions were counted on day 10. Drugs were given ip.

‡Where the mean number of lesions is equal to the average of $\sqrt{x^a}$ where x^a is the number of lesion per mouse. The virus rating (VR) is a ratio of the control over drug-treated.

TABLE 15.MOUSE WEIGHT AND TAIL LESIONS IN THE VACCINIA VIRUS MODEL AT FOUR VIRUS DOSES.

MEAN LESIONS (19.5 grams)	MEAN LESIONS (34.6 grams)	MEAN LESIONS TRANSFORMED ‡ $\sqrt{x^a}$ (19.5 grams)	MEAN LESIONS TRANSFORMED ‡ $\sqrt{x^a}$ (34.6 grams)
51.2±13.8	28.6±3.9	6.911±.926	5.30 ±.353
48.6±13.6	11.2±4.7	6.731±.908	2.957±.784
2.8±1.1	4.8±0.73	1.558±.305	2.216±.149
.4±0.3	.2±.2	.400±.245	.200±.2
0	0	0	

‡Where the mean number of lesions is equal to the average of $\sqrt{x^a}$ where x^a is the number of lesion per mouse. The virus rating (VR) is a ratio of the control over drug-treated. Lesions were counted on day 10. Drugs were given ip.

TABLE 16. DRUG EFFICACY REPORT: VACCINIA VIRUS IN THE TAIL LESION MODEL. COMPOSITE TEST RESULTS

AVS #	DRUG DOSE † (MG/KG)	CONTROL MEAN LESIONS‡ $\sqrt{x^a}$	DRUG MEAN LESIONS‡ $\sqrt{x^a}$	VR CONTROL ÷ DRUG	P (T-TEST PAIRED TWO-TAIL)
*1752	300	5.759	3.223	1.79	0.0016
*1985	300	5.759	2.553	2.26	0.0001
**3679	300	5.153	2.616	1.96	0.0007
***4224	300	6.165	1.739	3.55	0.0001
***2911	300	6.165	4.922	1.25	0.2129
***3607	300	6.165	3.923	1.57	0.0087

*Seven doses of drug were given, beginning one day before virus inoculation and continuing for five days after virus inoculation.
**Five doses of drug were given, beginning two days before virus inoculation and continuing for three day after virus inoculation.
***Eight doses of drug were given, beginning one day before virus inoculation and continuing for 6 days after virus inoculation.
‡Where the mean number of lesions is equal to the average of $\sqrt{x^a}$ where x^a is the number of lesion per mouse. The virus rating (VR) is a ratio of the control over drug-treated. Lesions were counted on day 10. Drugs were given ip.

SECTION FOUR: NONHUMAN PRIMATE PROTOCOL

A protocol was prepared in this laboratory for testing of an immunomodulator, Quinolinamine , AVS 1018, in the yellow fever primate model described in earlier years. The actual testing was delayed since imported Cynomolgous monkeys were impounded because of a potential outbreak of Ebola virus in these animals as judged by seroconversion of laboratory animal workers in another laboratory. The prepared protocol follows below.

NONHUMAN PRIMATE PROTOCOL

Title: Evaluation of the Effectiveness of Quinolinamine free base AVS 1018 [R-837 (S-26308)] Against Yellow Fever Virus Infection in Monkeys

Department: Yale University School of Medicine; Department of Epidemiology and Public Health

Division: Yale Arbovirus Research Unit

Study Director: Dr. Gregory H. Tignor, Yale School of Medicine
Dr. M. Kende, USAMRIID, Consultant

Investigators: Dr. Robert E. Shope
Dr. Feisha Zhao

Personnel: Mr. Ruben Cedeno

Animal Caretaker: Ms Susan Morgenstern

Management Data:

Initiation Date: May 20, 1990 (Day -1)
Termination Date: June 11, 1990 (Day 21)

Research Contract No. DAMD17-86-C-6042

Research Contract Title: Drug Development Against Viral Diseases

Project No: AVS 1018 (Continuation)

Lab Notebook No: Antiviral Drug Studies: Primates (AVS 1018)

Location: Rooms 600 and 600B, Laboratory of Epidemiology and Public Health, Yale University School of Medicine

Background:

AVS 1018 [R-837 (S-26308)] , is provided by 3M Riker, 3M Health Care Group, St. Paul, MN The rationale for use of this drug in primate tests with yellow fever virus is as follows.

Results in primates. This drug has been shown to be an immune response modifier. The subcutaneous toxic dose in monkeys (cynomolgous) is 5-6 mg/kg. On oral administration, the toxic dose is >200 mg/kg [2].

Results in the guinea pig model. AVS 1018 has been shown to have modest *in vitro* and *in vivo* activity against guinea pig CMV perhaps due to interferon induction [3], and to inhibit primary *in vivo* genital herpes [4]. AVS 1018 was protective against HSV-2 disease *in vivo* apparently by virtue of its immune up-regulating capacity resulting in enhanced cytokine production and cell-mediated immune responses in guinea pigs.

After intravaginal administration of a 1% suspension of AVS 1018-837 (50 µl/100g) to guinea pigs for five days, every 12 hours, beginning 12 hours after genital HSV-2 inoculation, vaginal virus replication was significantly reduced as compared to controls ($P<0.0001$). Treatment protected against primary disease and reduced recurrent genital HSV disease. Latent HSV-2 was not recovered from neural tissues of treated animals whereas there was an 83% recovery rate in placebo-treated recipients ($P=0.015$, Fisher Exact Test). Side effects of drug-treatment included mild fever, weight loss, and decreased water intake.

Immunologically, there was immediate interferon production, variable enhancement of either early or later cytolysis of HSV-2 targets, and early induction of HSV-2 induced PBMC proliferation and IL-2 production. ELISA and ADCC antibody were produced later and at lower levels[5]. In some AVS 1018-treated animals, HSV-2 replication was stopped so abruptly that no memory dependent immune functions resulted.

Results in the mouse model. Protection studies with a soluble HCl salt of the AVS 1018 in mice (CD-1 strain) showed that three drug doses (12.5 mg/kg) given subcutaneously on days 1, 6, and 11 after infection protected 76% of

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REFERENCES

[2]Personal communication from T. Monath, USAMRIID.

[3]Chen, M., Griffith, B.P., Lucia, H.L., and Hsuing, G.D. (1988) Antimicrob. Agents Chemother. 32, 678-683.

[4]Miller, R.L., Imberston, L.M., Retter, M.J. Schwartzmiller, D.S., Pecore, S.E. and Gerster, J.F. (1985) Inhibition of herpes simplex virus infections in a guinea pig model by S-26308. In: Program and Abstracts of the 25th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Minneapolis, Minnesota.

[5]Harrison, C.J., Jenski, L., Voychehovski, T., and Berstein, D.I. Antiviral Research, 10 (1988) 209-224.

CD-1 mice from Rift Valley fever virus (RVFV) challenge (250PFU's)[6]. Oral administration of 12.5 or 25 mg/kg doses was less effective. Viremia could not be detected in mice treated subcutaneously or orally with 12.5 mg/kg on days 1, 4, 10, and 13. Liver function enzymes and bilirubin values were elevated in RVFV-infected mice, but not in infected mice treated with 12.5 mg/kg of drug on days 1, 4, 10, and 13.

Three different oral vehicles which solubilized the free base of AVS 1018 were tested for efficacy in the RVFV mouse model. Oleic acid may have had a detrimental effect as a vehicle or the compound's solubility was not optimal in that vehicle. Lactic acid appeared to be suitable for both oral and subcutaneous administration because 68% and 54% respectively of treated mice survived following a dose of 12.5 mg/kg. AVS 1018 efficacy was increased when the free base was solubilized in 5% Tween-80. Oral administration of 12 or 6 mg/kg yielded 82 and 50% survivors respectively compared to 69 and 33% respectively with the HCl salt[7].

Purpose: To assess the antiviral efficacy of AVS 1018 (1-isobutyl-1H-imadazo [4,5-c] quinolin-4 amine) against yellow fever virus (YF).

Hypothesis: Quinolinamine therapy will inhibit YF virus replication *in vivo*.

Objectives:

1.0 To evaluate the antiviral efficacy of AVS 1018 using variable parameters of survival time and virus titer.

2.0 To obtain hematological, liver and renal function biochemical analysis in the treated and nontreated monkeys during YF virus infection.

3.0 To obtain evidence of immunological enhancement after drug treatment by measuring specific parameters of immune function in infected animals which are treated or mock-treated.

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[6]Kende, M., Lupton, H.W., and Canonico, P.G. Treatment of experimental virus infections with immunomodulators. Advances in the Biosciences. 55:51-63. 1988.

[7]Kende, M., Rill, W.L., Contos, M.J., and Canonico, P.G. Efficacy of a novel orally active immunomodulator, S-26308, against arbovirus infection. Antiviral Research 9:86, 1988.

Materials and Methods:

1. Animals (test model):

Ten healthy Ebola virus-free cynomolgous monkeys will be used for the study. Animals will be used only if they are free of HI antibodies to YF, dengue 1,2,3,4, and SLE virus and neutralizing antibodies to YF, Zika, WN, JE and Sepik viruses. Young adult monkeys will be used. The age will be verified by dendition. The monkeys should weigh 2-3 kg. The monkeys will be identified by chest tattoo and caged in appropriately marked squeeze cages in a biological containment laboratory. They will be fed twice daily (Purina Monkey Chow) and allowed water ad libitum. This diet will be supplemented with fresh fruit. General animal care will be in accordance with University Guidelines for Care of Nonhuman Primates as outlined in approved protocol #4804 filed with Yale University's Animal Care and Use Committee. The animal supplier will be the approved source, Charles River Primates, as arranged by the Yale University Division of Animal Care. Lot numbers of monkey chow used will be recorded during the course of the study.

2. Environmental control:

The animal rooms will be lighted with fluorescent lights and maintained on a 12-hr diurnal cycle. Room temperature and humidity will be regulated to 75°F and 50% respectively to avoid extreme fluctuations. Animal rooms and cages will be cleaned and sanitized prior to use. Cages will be cleaned once a day.

3. Test article (AVS 1016) and control article (Klucel):

Powered Quinolinamine (AVS 1016) will be used in this study. The compound is synthesized by 3M Riker, 3M Health Care Group, St. Paul, MN. The compound will be dissolved in 2% Klucel to a concentration of 100 mg/kg. The drug will be prepared fresh daily. The compound will be given by oral intubation according to recommendation of the manufacturers. An initial dose of 20 mg/kg will be given on day -1 followed by dose of 10 mg/kg on day 0 and other days as described below. A sample of the administered drug will be saved to give the supplier an opportunity to verify the drug concentration. The test articles, the vehicle, and the prepared solution will be stored in the refrigerator to minimize exposure to light and maintain the stability and sterility of the test article and the prepared solutions. Drug will be used within 30 minutes of preparation.

4. Virus.

YF virus, strain Asibi, was originally isolated from a 28 year-old male in Lagos and has been maintained in rhesus monkeys with an occasional passage by A. aegypti. In experiments with cynomolgous monkeys, three of four monkeys inoculated with Asibi virus from monkey 1225 died on days 3, 4, and 5. The seed virus is the 6th rhesus monkey and 1st mosquito passage. 1000 PFU in 0.2 mls will be administered I.m. in the forearm. This dose is usually lethal to cynomolgous monkeys in 3-5 days.

5. Experimental Design:

a. Monkeys will be assigned to one of three experimental groups using a calculator program for random number generation. The monkeys will be weighed on

days -1, 0, +2, +4, +6, +8, +10, +12, +14.

b. On day -1, five monkeys assigned to Group 1 will be given 20 mg/kg of AVS 1018. On day 0, these monkeys will receive 1000 PFU YF virus. Immediately after virus inoculation, monkeys will be given 10 mg/kg of drug by oral intubation and for 7 doses thereafter the monkeys will receive 10 mg/kg AVS 1018 by oral intubation. Drug will be given on day -1, 0, +2, +4, +6, +8, +10, +12, +14. Animals will be bled 6-8 hours after drug administration at the recommendation of the manufacturer.

c. On day -1, three monkeys assigned to Group 2 will be given placebo. On day 0, these monkeys will receive 1000 PFU YF virus. Immediately after virus inoculation, monkeys will be given placebo by oral intubation and for 7 doses thereafter the monkeys will receive placebo. Placebo will be given on day -1, 0, +2, +4, +6, +8, +10, +12, +14. The placebo is the drug vehicle: 2% Klucel.

d. On day -1, two monkeys assigned to Group 3 will be given 20 mg/kg of AVS 1018. On day 0, these monkeys will be given 10 mg/kg of drug by oral intubation and for 7 doses thereafter the monkeys will receive 10 mg/kg AVS 1018 by oral intubation. Drug in Klucel (2%) will be given on day -1, 0, +2, +4, +6, +8, +10, +12, +14.

e. All monkeys will be sedated with 0.1 ml of a mixture of equal parts of ketamine HCL (100mg/ml) and atropine sulfate (1/120 Gr) injected i.m. into the muscle masses of either hind leg to facilitate bleedings. Nine mls of blood will be drawn with needle and syringe on the following days: -3, -1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 20. The blood will be divided as outlined in f, g, h and i. The experimental protocol is summarized in the following chart.

TREATMENT SCHEDULE FOR AVS 1018

Group designation	Number of monkeys	YF virus Day 0	Treatment	Bleedings on day -3,-1,0,1,2, 3,4,5,6,7,8, 10,12, 14,20
1	Five	+	Drug	+
2	Three	+	Placebo	+
3	Two	-	Drug	+

f. 0.5 ml aliquots of blood for hematology on days -3,-1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 20 will be placed in a NUNC brand tube containing EDTA (0.1ml of 10% EDTA in water; the final concentration of EDTA after addition of blood is 1.67%.). Samples will be sent on wet ice on the day drawn by Federal Express courier to USAMRIID. The following determinations will be done: total RBC, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count, platelet count, total and differential leukocyte counts. Hematologic analysis will be conducted by the Clinical Laboratory in the hot suite at USAMRIID.

g. 0.5 ml aliquots of plasma will be held on dry ice for shipment by Federal Express to USAMRIID on days -3,-1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 20. One ml of whole blood will be put in a silicone coated NUNC brand tube containing 0.05 ml of Na-citrate (3.8% (0.129M) in buffered citrate solution (16.0 mg $Na_3Citrate\ 2H_2O$ and 2.1 mg. Citric Acid·$1H_2O$). After centrifugation, the plasma will be frozen in a dry ice-methanol bath. After shipment to Fort Detrick, the plasma will be assayed for 1 stage prothrombin, activated partial thromboplastin time (APTT), and Factor VIII.

h. 2.0 mls of whole blood will be added to a commercially purchased FDP tube (Ortho Diagnostic Systems) which will then be held frozen on dry ice for shipment to Fort Detrick. Fibrin degradation products will be determined by the Clinical Laboratory in the hot suite at USAMRIID.

i. 1.0 ml aliquots of serum from approximately 3 mls of blood on days -3,-1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 20 will be frozen and held for shipment by Federal Express to USAMRIID. This serum, divided into two different 0.5ml aliquots, will be used for IL-1, IL-2, TNFα, SGOT, SGPT, Bilirubin, BUN and creatine assays in the hot suite at USAMRIID.

j. 1.0 ml aliquots of serum from approximately 3 mls of blood on day -3,-1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 20 will be held at YARU at -70°C. Viremia will be determined for groups 1, 2, and 3. The serum will also be assayed for neutralizing antibody in tissue culture. Serum samples drawn 6-8 hours after administration of drug (or placebo) on days -1, 0, +2, +4, +6, +8, +10, +12, +14 will be tested for α/β interferon in bioassay. INF-γ determinations will be done by RIA.

k. Sick animals will not be held for observation but will be exsanguinated under pentabarbitol sedation immediately upon onset of clinical signs. Tissues collected at necropsy will be assayed for viral titers. Sample containers and histological slides will be labeled with animal number, study number, type of sample, date of collection, and technicians initials.

l. Animals will be observed for general health and mortality for 20 days. Clinical observations will be recorded in the raw data notebook. These data will be transferred to a computer disk. Necropsy will be performed on dead monkeys as soon as possible and a small portion of the liver, brain, lung, heart, kidney, thymus, spleen and mesenteric lymph nodes will be frozen for virus titration and a sample from these organs will be preserved in 10% buffered formalin for histology. A complete record of the dead animals will be recorded in the study notebook. Surviving monkeys will be used for other experimental protocols or will be killed by pentabarbitol injection. Their serum will be tested for antibodies to viral agents used in neighboring animal holding rooms.

m. Body weight, hematology, enzyme levels, and serum chemistry data will be analyzed by comparison of the groups using analysis of variance.

n. All procedures will be performed according to standard operative procedures used either at YARU or in the Division of Antiviral Studies.

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