A NEW ANTIVIRAL AGENT DESIGNATED 6-MFA FROM *ASPERGILLUS FLAVUS*

III. AMPLIFICATION OF ANTI-SEMLIKI FOREST VIRUS ACTIVITY OF 6-MFA BY CYCLOHEXIMIDE TREATMENT IN MICE

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Investigations on the anti-Semliki Forest virus activity of a new antiviral agent (designated 6-MFA) obtained from fungus *Aspergillus flavus*, strain 6-MFA, show that when low doses of 6-MFA, having alone little effect, are suitably combined with cycloheximide, antiviral activity in mice may be enhanced 9- to 10-fold.

MAHESHWARI *et al.*[1] and MAHESHWARI and GUPTA[2] reported that 6-MFA, a new antiviral agent isolated from *Aspergillus flavus*, in culture is effective against three viral infections namely Semliki Forest, Chikungunya and neurovaccinia virus, in that order, in mice. The method of mold culture, fermentation, extraction of 6-MFA and its bio-assay have been reported[3]. The 6-MFA substance is obtained as pale white powder and is thermolabile, Seitz-filterable, non-dialyzable, sedimentable. Samples of 6-MFA have been seen under electron microscope to contain pentagonal virus-like particles[4].

With a view to elucidate the mode of action of 6-MFA we have now studied the effect of low doses of 6-MFA in presence of cycloheximide, an inhibitor of protein synthesis in mice and report an enhancement of the antiviral effect.

Materials and Methods

Mice 35-Days old Swiss-CDRI strain mice weighing 16~18 g were used.

Virus The Semliki Forest Virus (SFV) of SMITHBURN and HADDOW[5] obtained from ATCC, U. S. A. was maintained in mice intracranially. Brain homogenate (10% w/v) was prepared in HANKS BSS. Supernatant obtained by centrifuging the homogenate at 3,000 r.p.m. for 30 minutes was kept at −10°C in aliquots of 1 ml each. Dilution of the virus was prepared in HANKS just prior to use. LD_{50} was calculated according to REED and MUENCH formula[6] and found to be $10^{-6.62}$, 0.5 ml per mouse subcutaneously (s. c.).

6-MFA It was prepared by acetone treatment of the crude filtrate as described, and the ED_{50} was 36 mg/kg body weight in mice[3].

Cycloheximide It was obtained from Upjohn Company, Kalamazoo, Michigan, U. S. A. Maximum tolerated dose of cycloheximide worked out to be about 88 mg/kg body weight in our mice. Above this dose treated mice died within 24 hours.

Antiviral assay 6-MFA was given to mice by intraperitoneal (i.p.) route and cycloheximide also administered i. p., prior, after or along with 6-MFA. Mice were challenged with 100 LD_{50}, SFV, 0.5 ml per mouse s. c. 24 hours after the administration of 6-MFA.

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Results

In the first experiment, a dose of 6-MFA (117 mg/kg body weight) which protected 100 % of the test mice against SFV infection was administered 2 hours before injection of cycloheximide (88 mg/kg body weight). It was seen that 50 % of the animals died of toxicity symptoms within 12 hours, suggesting an additive toxic effect because the same concentration of cycloheximide or 6-MFA alone produced no such mortality. Such additive toxic effect had been observed by YOUNGNER and HALLUM[7] in mice treated with cycloheximide and then given poly I : C at doses which by themselves did not have any overt toxic effect.

In the next experiment cycloheximide was given 1 hour before, after or along with 6-MFA, and animals challenged 24 hours later with SFV. Results (Table 1, experiment I) show that cycloheximide (56 mg/kg) treatment enhanced significantly the antiviral activity of 6-MFA (14 mg/kg). As many as 71~87 % of mice were protected as against only 25 % in the 6-MFA and cycloheximide controls. This test was repeated in mice treated with a still lower dose of 6-MFA (5.6 mg/kg). Similar enhancement of the antiviral efiect of 6-MFA by cycloheximide treatment was observed. However, the effect of cycloheximide appeared to wear off with time, more so, when cycloheximide was given before or along with 6-MFA, but not when cycloheximide followed 6-MFA. This test showed that long-term enhancement of antiviral activity of 6-MFA was more significantly achieved when cycloheximide was injected after 1 hour of the administration of 6-MFA (Table 1; experiment II).

Further test was performed in which interval between 6-MFA (5.6 mg/kg) administration and cycloheximide (56 mg/kg) injection was varied from 1 hour to 4 hours. Results presented in Table 2 show that in 82 mice, given both cycloheximide and 6-MFA, the proportion of animals surviving virus challenge ranged from 38 to 89 %, compared to 0, 10 and 20 % survivors in the three controls *viz.* virus, 6-MFA, and cycloheximide, respectively. Second, when cycloheximide was given prior to 6-MFA even though initial response was encouraging, persistence of enhanced antiviral activity was

Table 1. Effect of cycloheximide treatment on antiviral activity of 6-MFA

Treatment	Experiment I*		Experiment II**			
	10 days observation		8 days observation		15 days observation	
	Survivors(*)	Survival (%)	Survivors	Survival (%)	Survivors	Survival (%)
6-MFA alone (control)	2/8	25	2/10	20	0/10	0
Cycloheximide alone (control)	2/8	25	3/10	30	0/10	0
Cycloheximide 1 hour before 6-MFA	7/8	87	9/10	90	1/10	10
Cycloheximide along with 6-MFA	7/8	87	7/10	70	2/10	20
Cycloheximide 1 hour after 6-MFA	5/7	71	4/9	45	4/9	45
Buffer saline (control)	0/8	0	0/10	0	—	—

* 6-MFA in liquid form, suspended in buffer at the rate of 0.25 ml/mouse, equivalent to approximately 14 mg/kg, body weight; cycloheximide 56 mg/kg

** 6-MFA in freeze-dried form, suspended in buffer at the rate of 5.6 mg/kg; cycloheximide 56 mg/kg

(*) mice surviving/total number

Table 2. Effect of varying the time of administration of cycloheximide on the antiviral activity of 6-MFA

Cycloheximide treatment*	8 Days observation		15 Days observation		
	Survivors**	Survival (%) (a)	Survivors	Survival (%) (b)	Drop in antiviral activity (*) (%)
4 hours before 6-MFA	7/9	78	1/9	11	67
3 hours before 6-MFA	6/9	66	1/9	11	55
2 hours before 6-MFA	6/9	66	1/9	11	55
1 hour before 6-MFA	8/9	89	1/9	11	78
0 hour along with 6-MFA	4/8	50	2/8	25	25
1 hour after 6-MFA	6/8	75	5/8	62	13
2 hours after 6-MFA	3/8	38	2/8	25	13
3 hours after 6-MFA	5/11	46	1/11	9	37
4 hours after 6-MFA	7/11	63	2/11	18	45
Control (6-MFA only)	1/10	10	0/10	0	100
Control (cycloheximide only)	2/10	20	0/10	0	100
Control (buffer saline only)	0/10	0	—	—	—

* 6-MFA 5.6 mg/kg; cycloheximide, 56 mg/kg
** mice surviving/total number
(*) Drop in antiviral activity=(a−b)

poor. Enhancement of antiviral activity did not wear off substantially if cycloheximide was given 1 to 2 hours after 6-MFA. Results in experiment I essentially agree with that observed in experiment II, of Table 1.

Discussion

Several workers[8~14] have reported that addition of cycloheximide (an inhibitor of protein synthesis) can enhance interferon production in cultured tissue cells pretreated with a variety of inducers. Because of a possible clinical significance of this, we have studied the anti-Semliki Forest virus activity of a fungal growth product 6-MFA in presence of cycloheximide in intact mice. A total of 218 mice was employed in three experiments. The antiviral activity (% of mice protected, of 6-MFA, given at the rate of 5.6 mg/kg could be amplified 9- to 10-fold by simultaneous treatment of mice with cycloheximide (56 mg/kg body weight). On the basis of results presented it would appear that the extent and duration of enhancement of the antiviral effect of 6-MFA by cycloheximide vary with the time of its administration.

We have not determined the concentration of any viral inhibitor (interferons) in sera or body tissue of mice treated with 6-MFA as yet. It may be that enhanced mouse protective action achieved by combined treatment of 6-MFA and cycloheximide reported here is mediated by accelerated production of interferon, though there could be other explanations also for such enhancement[15]. However, a direct sensitive bioassay of interferon would settle this point.

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