

THE EFFECT OF INTERFERON ON FLAVIVIRUSES *IN VITRO*: A PRELIMINARY STUDY

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INTRODUCTION

Since the discovery of Interferon (IFN) (Isaacs and Lindenmann, 1957) the clinical potential of IFN has grown enormously especially during the past several years (Stewart, 1981). IFN is a family of inducible glycoproteins with molecular weight of 20,000-40,000 daltons, produced by eukaryotic cells in response to viral infections or a number of inducers. IFN can inhibit the replication of a wide variety of viruses, and it is mainly host species-specific. At present, 3 types of IFN are known, i.e. IFN α , IFN β and IFN γ . Human IFN α can be produced on a large scale from blood leukocytes or lymphoblastoid cell culture, while IFN β can be obtained massively from fibroblast cell culture. IFN γ which is produced by lymphocytes during immune response, has not yet been available in sufficient quantity for clinical studies. Recently, IFNs have been suggested as potential agents against some types of cancer (Krim, 1980; WHO, 1982).

In many parts of the world, Flavivirus group of viruses have caused diseases of high morbidity and mortality. In Thailand and other countries in Southeast Asia, we have encountered public health problems due to Dengue Haemorrhagic Fever (DHF) and Japanese Encephalitis (JE) during the past several years. DHF has been recognized as an endemic disease of public health importance in Thailand, Philippines, Singapore, Malaysia, Vietnam, Burma and Indonesia. DHF is caused by an arbovirus, with haemorrhagic

phenomenon of dengue fever, and occurs as endemic and epidemic forms especially during rainy seasons. The causative agents are dengue viruses serotypes, 1, 2, 3 and 4 (Flavivirus) and Chikungunya virus (Alphavirus), and the disease is transmitted by *Aedes aegypti* mosquitoes breeding in artificial containers in close relation to human inhabitats. Cases are usually children aged below 9 years especially between 3-6 years (Thongcharoen, 1977).

JE is one of the serious arthropod-borne viral infection, now recognized as an endemic disease in Southeast Asia particularly in Thailand. It is characterized clinically by fever associated with central nervous system involvement resulting in severe headache, convulsion, paralysis, coma and death. Clinical manifestations range from mild febrile episodes with or without meningo-encephalitis symptoms to fulminating febrile with severe encephalitis and sequelae, and death finally. The disease is transmitted by *Culex tritaeniorhynchus* and other species of mosquitoes which breed readily in rice paddy fields and other rural types of water-beds, thus presenting difficult problems in their control. In Thailand, sporadic cases have been reported throughout the year, and most outbreaks occur during the rainy season (May-October). Most of the patients are children aged between 1-14 years. The mortality rate of the disease is rather high, about 19.5%-31.3% in general (Grossman *et al.*, 1973; Igarashi *et al.*, 1983).

There is at present no specific drug for the treatment of either DHF or JE. It is worthwhile to study on the use of interferon, a new antiviral agent on these 2 viral infections. However, since recently IFN α and IFN β have been available for laboratory tests in our institutions, our objective is to study their effect on dengue virus and JE viruses *in vitro* in our laboratories. If the *in vitro* studies should prove successful, IFN can be used as potential drug for the treatment of DHF and JE patients in the near future.

MATERIALS AND METHODS

The studies: The effect of human IFN α and IFN β on dengue virus type 2 in our laboratories was determined by (1) plaque reduction test in LLC-MK₂ monolayer cell culture, and (2) infected cell depressing effect (ICDE) in C6/36 cell culture determined by direct fluorescent antibody technique, while those on JE viruses were investigated by 50% inhibition of CPE in LLC-MK₂ monolayer cell culture (Ferreira *et al.*, 1979).

LLC-MK₂ cells (Rhesus monkey kidney cell line) were grown in medium 199 supplemented with 15% calf serum, penicillin and streptomycin. For experiments, cells were seeded as 1×10^5 cells/ml/tube; a good sheet of monolayer in a tube was formed in 3-4 days which was ready for use.

C6/36 cells (*Aedes albopictus* mosquito cell line) were grown in medium RPMI supplemented with 10% foetal calf serum, penicillin and streptomycin. For experiments, cells were seeded as 1×10^4 cells/ml/tube which were ready for use in 3-4 days.

The viruses:

Dengue virus type 2, prototype New Guinea C strain (NGC) obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS, Bangkok-Department of Viro-

logy) was propagated in suckling mice and then passed to C6/36 cell culture.

JE virus Japanese prototype JaGAR strain was obtained from the National Virus Research Institute, Department of Medical Sciences, Ministry of Public Health of Thailand. The virus was propagated in suckling mice and then adapted to LLC-MK₂ cell culture.

JE virus Vip local strain was isolated from the brain of a fatal case of JE in Siriraj Hospital and kept in the Division of Virology, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University. It was propagated in suckling mice for one passage and then passed to LLC-MK₂ cell culture for 2 passages. All viruses were titrated by plaque assay and CPE assay in LLC-MK₂ cell culture.

Interferon preparations:

IFN α is human lymphoblastoid cell interferon (Wellcome Foundation Ltd. London through Dr. David Warrell, Wellcome/Mahidol University/Oxford Medical Research Programme, Bangkok), Lot. CIN/14, CT3/0136 (9.1 mega units in 1 ml vial).

IFN β is human fibroblast interferon known as "Frone" produced by Inter-Yeda Research and Development Co. Ltd., Israel and supplied by Sero Pharmaceutical Co. Ltd., Rome (1,000,000 I.U./ml vial) and Union Medical (Thailand) Co. Ltd., Bangkok.

Interferon Titration:

Inhibition of CPE in monolayer cell culture: The interferon preparation was diluted to ten fold dilution in maintenance medium. M199 supplemented with 2% calf serum and antibiotics from 10^{-1} to 10^{-5} dilution. A 0.1 ml amount of each dilution was added into two tubes of LLC-MK₂ cells monolayer.

After 12 hours incubation at 37°C, the medium was removed, and 0.1 ml of approximately 100 TCID₅₀ of virus suspension diluted in maintenance medium without serum was added to the culture tubes. The tubes were then incubated at 37°C 1 hour for viral absorption, and then the maintenance medium was added and the tubes were re-incubated again. CPE was observed daily and the interferon titer was expressed as the reciprocal of the highest dilution which showed 50% inhibition of CPE.

Plaque reduction test : The interferon was prepared in the same dilution as stated in the CPE Inhibition test above. Plaque reduction test was performed as in the standard plaque method (Vithanomsat *et al.*, 1983). Before inoculation of dengue virus into LLC-MK₂ cell culture the IFN treated cells were incubated at 37°C overnight. The test were read after staining with neutral red on day 6 after virus inoculation.

The interferon titer was expressed as the reciprocal of the highest dilution which showed 50% of plaque reduction.

Immunofluorescent assay : The interferon was diluted in the same manner as described above. The C6/36 cell monolayer was treated with various dilution of IFN for 12 hours at 28°C. The appropriate dose of dengue virus was added, then the cell culture was incubated at 28°C for 4 days. The cells in cover slip treated with interferon virus were then removed from Leighton tubes and fixed with cold acetone. They were processed by direct fluorescent antibody staining, using anti-dengue 2 fluorescent isothiocyanate conjugate and counter stained with Evans blue. Fluorescent foci were read under a fluorescent microscope.

The interferon titer was expressed as the reciprocal of the highest dilution which cause 50% reduction of fluorescent foci, or 50% infected cell depressing dose (ICDD₅₀)

RESULTS

The results of the effect of Interferon on Dengue virus type 2 as determined by the plaque reduction test in LLC-MK₂ cell culture is shown in Table 1.

The IFN α showed titer of 10^{2.5} in 50% reduction of Dengue virus type 2 plaque, while the IFN β showed no effect even at titer of 10¹.

Table 1
Results of the effect of IFN α and IFN β on Dengue virus type 2 in LLC-MK₂ cell culture.

IFN	Dengue virus type 2	Titer of IFN	Plaque reduction test 50%
α	New Guinea C strain	10 ^{2.5}	+ ve
β	New Guinea C strain	10 ¹	- ve

The results of the effect of Interferon on Dengue virus type 2 as determined by the fluorescent antibody technique in C6/36 cell culture is shown in Table 2.

Table 2
The inhibiting effect of IFN α and IFN β on Dengue virus type 2 fluorescent foci in C6/36 cell culture.

IFN dilution	No. of fluorescent foci (200 cells count)		% reduction	
	IFN α	IFN β	IFN α	IFN β
10 ⁻¹	16	75	65	0
10 ⁻²	38	74	49	0
10 ⁻³	74	76	0	0
10 ⁻⁴	72	78	0	0
10 ⁻⁵	71	78	0	0
Virus control	75	75	0	0

Titer of ICDD₅₀ of IFN α equal to 10^{1.96}

The results were more or less similar to those of the plaque reduction assay. The IFN α showed titer of $10^{2.7}$ with 50% infected cell depressing dose, while the IFN β showed no depressing effect at titer of 10^1 .

The effect of Interferon in inhibition of JE virus CPE in LLC-MK₂ cell culture is shown in Table 3.

The IFN α at titer of 10^5 and $10^{4.5}$ gave 50% inhibition of JE virus Japanese prototype JaGAR strain and JE virus Vip local strain respectively. The IFN β showed different effects on JE virus JaGAR strain and Vip local strain. The IFN β at a concentration of 10,000 I.U. showed 50% inhibition of the JaGAR strain, while a concentration of 10 I.U. could affect the Vip local strain.

The IFN α and IFN β at dilution of 10^{-1} showed no toxicity effect on either the LLC-MK₂ cell culture or the C6/36 cell culture.

Table 3.

Results of the effect of IFN α and IFN β on JE viruses in LLC-MK₂ cell culture.

IFN	JE virus strain	Titer of IFN I.U./0.1 ml	
α	JaGAR	10^5	9.1
α	Vip	$10^{4.5}$	28.8
β	JaGAR	10^1	10,000
β	Vip	10^4	10

DISCUSSION

The present study in our laboratories is only a preliminary investigation on the effect of human Interferon α and Interferon β on one strain of Dengue virus and 2 strains of JE viruses. The results revealed that generally JE viruses were more susceptible to these 2 Interferons antiviral agents than Dengue virus, as shown in Table 1, 2 and 3. Further studies are required in order to expand these

findings and to find the specificity of each interferon on individual viruses.

In assessing *in vitro* sensitivity tests of the viruses to interferon, the plaque reduction test in LLC-MK₂ monolayer cell culture and the immunofluorescent assay on C6/36 cell culture and also the inhibition test on CPE in LLC-MK₂ monolayer cell culture proved to be highly sensitive. Thus these 3 methods will be used later for further studies on the effect of interferon on viruses in our laboratories.

The results of these studies have indicated that a clinical trial of interferon on Japanese encephalitis would be given priority if it becomes available in Southeast Asia in the near future.

SUMMARY

The preliminary results of our study *in vitro* on the effect of Interferon on Flaviviruses showed that Interferon α and Interferon β were more effective on JE viruses Vip local strain and JaGAR strain, but less on Dengue virus type 2 strain. However, the effect of these 2 interferons on the 2 strains of JE viruses were still variable which need further investigations. The JE virus Vip local strain seemed to be more susceptible to interferon than the Japanese prototype JaGAR strain. Thus, the *in vivo* trial on JE disease in Thailand is strongly recommended.

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