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INHIBITION OF INFLUENZA VIRUS A/WSN REPLICATION BY A TRYPSIN INHIBITOR, 6-AMIDINO-2-NAPHTHYL p-GUANIDINOBENZOATE

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A trypsin inhibitor, 6-amidino-2-naphthyl p-guanidinobenzoate (FUTHAN) reduced both the number and size of plaques of influenza virus A/WSN/33 (H1N1) that can grow without trypsin treatment in MDCK cells. The resulting virus particles with uncleaved hemagglutinin(HA) in the presence of FUTHAN was activated to produce infectious virions by trypsin treatment. Uncleaved HA of WSN virus grown in the presence of FUTHAN was found to be accumulated by protein analysis of WSN virus labeled biosynthetically with [^{35}S]-methionine. It was strongly suggested that FUTHAN inhibited viral replication by preventing proteolytic cleavage of HA.

A chemically synthesized substance,6-amidino-2-naphthyl p-guanidinobenzoate (designated FUTHAN, Fig-1) is a potent and specific inhibitor of trypsin-like serine protease (1,2,3) and has been used clinically for acute pancreatitis.

Ordinarily, influenza virus infection in humans causes only mild or moderate disease because primarily epithelial cells of the upper respiratory tract are involved. Especially, the trypsin-like endoprotease derived from host cell origin is essential to produce the infectious virions where HA has been cleaved (4). On usual replication of influenza virus in cell culture, it is indispensable to add trypsin to the cell culture to produce infectious particles. The virus strain that can grow in cell culture without trypsin treatment is very useful in evaluating the effect of trypsin inhibitors on the replication of the virus. Influenza virus A/WSN/33 (H1N1) can grow in MDCK cells without adding trypsin. Therefore, we studied the effects of trypsin inhibitor FUTHAN on the plaque formation and HA biosynthesis of WSN virus in MDCK cells.

Materials and Methods

Virus and cells Influenza virus A/WSN/33 and MDCK cells were kindly given by Dr. K.Nakajima (the Institute of Medical Science, University of Tokyo Tokyo,

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Abbreviations: HA, hemagglutinin. PFU, plaque forming unit.

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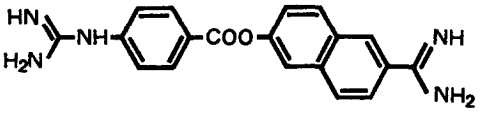

Figure 1. Chemical structure of FUTHAN.

Japan). The WSN strain was grown in MDCK cells. Plaque assay and HA titration were carried out according to standard procedure(5).

Purification of virus and analysis of viral proteins Virus was collected from cell culture medium and purified on sucrose gradient as described previously (6,7). For radioactive labeling of viral proteins, confluent monolayers of MDCK cells were grown in Dulbecco's modified MEM. After virus infection the cells were incubated with cold methionine-free Eagle's MEM containing 5μCi of [^{35}S]-methionine (specific activity >1000mmole)/ml and 0.1% (W/V) bovine serum albumin. Virus was harvested after 18 hr, cleared of cellular debris by centrifugation at 4000 g for 20 min at 4 C, and purified through a 60%-30% sucrose step gradient at the interface of the two sucrose concentrations. For gel electrophoresis (8), slab gels were analyzed by fluorography (9).

Results

Inhibition of plaque formation by FUTHAN

Table-1 shows the effect of FUTHAN on the plaque formation of WSN virus (H1N1) in MDCK cells. There was a dose-dependent reduction in plaque formation. Both the number and size of plaques decreased in the presence of FUTHAN. The plaque formation in MDCK cells was completely inhibited by FUTHAN at a concentration of 10 μg/ml without any cytotoxicity.

Effect of trypsin on WSN virus grown in the presence of FUTHAN

The mechanism by which FUTHAN inhibits infection or plaque formation in MDCK cells was investigated by studying the effect of trypsin on infectivity of WSN virus. Confluent monolayers were infected with WSN virus at a multiplicity of approximately 10. Sixteen hours after infection, virus either were left untreated or were treated with trypsin, HA titers and PFU of the supernatant fluids were measured. The results, summarized in Table-2, demonstrated that trypsin treatment of virus grown in the presence of FUTHAN

Table-1. Plaque number reduction of WSN virus in the presence of FUTHAN

Inhibitors	Inhibition(%)	Plaque size(mm)
None (Control)	0	2-3
FUTHAN (μg/ml)		
1.25	10	1-2
2.5	29	<1
5.0	62	Pin hole
10.0	100	Not detected

FUTHAN was added to the overlayed agar at the indicated concentration. The agar was removed 3 days after virus infection. MDCK cell sheet was fixed and stained with 2%(W/V) crystal violet solution in 50%(V/V) ethanol and then the number and size of the plaques were measured. The number of plaques in Control cultures was about 80 on an average.

Table-2. Activation of virus infectivity by trypsin treatment after growth in the presence of FUTHAN

	trypsin treatment	HA(unit/ml)	PFU/ml (MDCK cells)
Control	-	128	1.1×10^6
	+	128	1.4×10^6
FUTHAN(10μg/ml)	-	128	1.6×10^5
	+	128	9.6×10^5

MDCK cells were infected with WSN virus at MOI 10. Sample fluids were obtained 16 hours after infection. After 10^4 fold dilution to cancel out the effect of FUTHAN on plaque formation, the diluted solution was treated without or with 10μg/ml trypsin for 30 min at 37 C and assayed in MDCK cells. The original infectivity (PFU/ml) is shown above.

(10μg/ml) resulted in approximately a six fold increase in infectivity in MDCK cells. The results suggested that the failure of WSN virus to produce plaques in MDCK cells is related to the production of a high proportion of noninfectious virions containing uncleaved HA.

Inhibition of cleavage of WSN virus HA by FUTHAN

The proceeding observation suggested that WSN particles containing uncleaved HA were produced in MDCK cells in the presence of FUTHAN. To test this possibility, we examined directly the protein patterns produced on SDS-polyacrylamide gels by WSN virus grown in the presence or absence of FUTHAN. Fig.2A shows the protein patterns of WSN virus grown in the absence or presence of FUTHAN. Densitometric profiles in Fig.2B (a,b) clearly demonstrate that with WSN virus grown in MDCK cells a relatively large amount of the HA was present in the cleaved form. In contrast, in the presence of FUTHAN the uncleaved HA was found to be accumulated and at the same time the cleaved fragment HA1 apparently decreased compared with that in Control. From these results it was strongly suggested that FUTHAN inhibited viral replication by preventing proteolytic cleavage of HA.

Discussion

Post-translocational proteolytic cleavage of the viral HA by trypsin-like cellular endoprotease derived from host cell origin is essential for infectivity, spread of the virus in a host cell and pathogenicity (10). Usually, infectious virus cannot be produced in host cells where an appropriate enzyme for cleavage of HA is not present. Infectivity is recovered by treatment of noninfectious virions with trypsin in vitro (11).

We have investigated the effects of FUTHAN on the replication of WSN virus. We clearly showed that cleavage of the HA was inhibited at the concentration of about 10μg/ml which still allowed viral protein synthesis and formation of virus particles. Also, we demonstrated that most of WSN virus HA grown in the

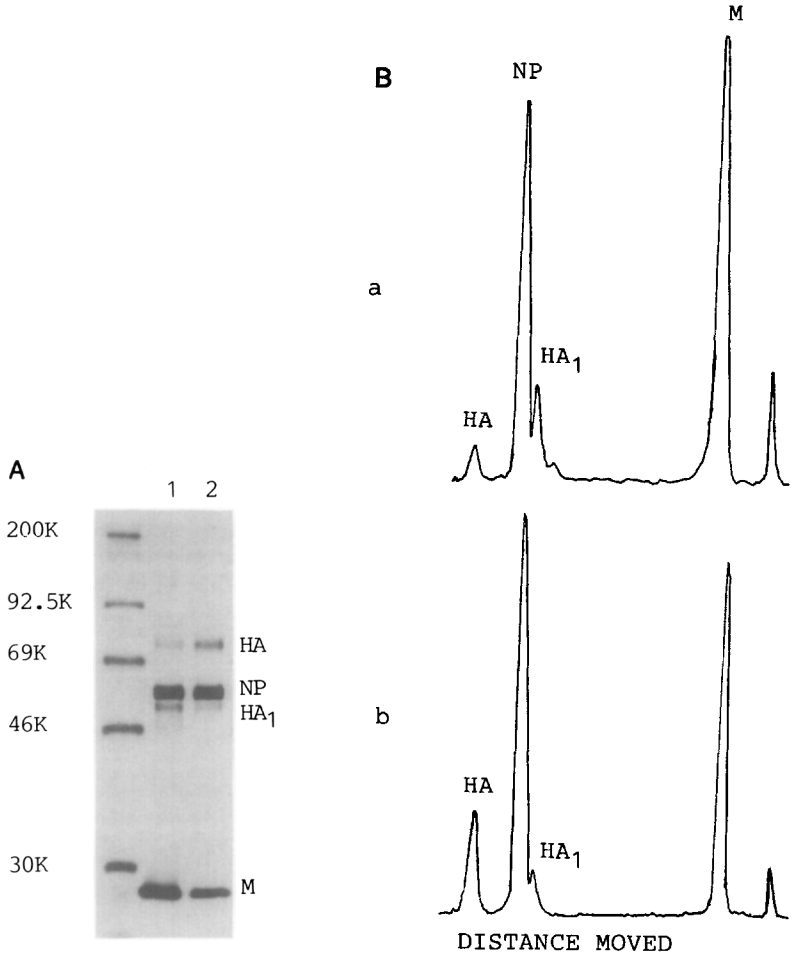

Figure 2. Inhibition of proteolytic cleavage of hemagglutinin(HA) of WSN virus by FUTHAN. A, Virus was grown in MDCK cells at MOI 10 in the absence (lane 1) or presence (lane 2) of 10μg/ml FUTHAN. [^{35}S]-methionine-labeled WSN virus was purified by discontinuous ultracentrifugation and disrupted with SDS. Proteins were separated on a 12.5% polyacrylamide slab gel and analyzed by fluorography. HA, uncleaved hemagglutinin; NP, nucleoprotein; HA1, cleaved fragment of HA; M, membrabe protein. B, Densitometric profiles of the developed X-ray films in the absence (a) or presence (b) of FUTHAN.

presence of FUTHAN remains uncleaved by protein analysis. Although it is not evident whether FUTHAN can pass through the cytoplasmic membrane of the target cells and cause elevation of the lysosomal or endosomal pH which results in inhibition of the cleavage of the virus HA, our observations indicate the possibility that FUTHAN inhibits the activity of the cellular processing enzyme that activate virus HA. The HA cleavage protease from a host cell has not been identified. At present we do not have direct evidence in support of the hypothesis that FUTHAN directly inhibits the activity of the HA cleavage enzyme. The answer to the question will be made after the isolation and purification of the activation enzyme from host cell origin.

Acknowledgment

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