MODE OF ACTION OF TRICHOTHECIN ON MULTIPLICATION OF NEWCASTLE DISEASE VIRUS IN CULTURED CELLS

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Trichothecin (1.6 mcg/ml) completely inhibited multiplication of Newcastle disease virus in cultures of chick embryo fibroblasts infected with 50 plaque forming units/cell; while at lower concentrations, where complete inhibition was not observed, a dose-response effect was shown by a lengthening of the lag time before the beginning of hemagglutinin synthesis. Trichothecin had no effect on free virus particles or on viral adsorption to host cells. The antibiotic exerted its activity whenever it was added during a viral growth cycle, and the inhibitory action was reversed upon removal of trichothecin by washing. Protein synthesis was promptly suppressed after addition of trichothecin (3.0 mcg/ml), but actinomycin D-insensitive RNA synthesis proceeded in the presence of the antibiotic. The RNA was analysed on a methylated albumin-kiselguhr column.

In searching for antiviral antibiotics of microbial origin, we found that trichothecin produced by *Trichothecium roseum* has a remarkable inhibitory effect on multipication of some DNA and RNA animal viruses[1]. To clarify the mechanism of action on viral growth, some of the effects of the antibiotic were examined employing a system with Newcastle disease virus (NDV) and chick embryo fibroblasts (CEF) virus cells. Trichothecin was found to be a potent inhibitor of protein synthesis. This and other properties of the compound will be described below.

Materials and Methods

Virus-cell system: The Miyadera strain of NDV was grown in cultured CEF according to methods reported previously[2].

Infection of CEF: Monolayer cultures of CEF in test tubes (10×200 mm) or Petri dishes (90 mm in diameter) were washed twice with precooled (5°C) medium, infected with NDV at an input multiplicity of 50 plaque forming units (PFU)/cell, and were allowed to stand at 5°C for 2 hours for viral adsorption. Unadsorbed virus was removed by washing three times with prewarmed (38.5°C) medium and fresh medium was added. Viral growth was followed at 38.5°C.

Dose-response experiments: NDV-infected CEF monolayers in test tubes were refed with fresh prewarmed medium containing trichothecin, and NDV growth was followed by titration of hemagglutinin units (HAU) and/or PFU after freezing-and-thawing duplicate tubes at each drug concentration and sampling time in a dry ice-acetone bath.

Addition time effect: Trichothecin was added to infected (multiplicity of 50 PFU/cell) cell sheets in test tubes at various times after infection during one viral growth cycle and

viral multiplication was followed by tritration of HAU.

Recovery from inhibitory action of trichothecin: After various treatment periods of infected cells with trichothecin (5.0 mcg/ml), the antibiotic was removed by washing the cell sheets three times and the restoration of viral growth was examined by titration of PFU and/or HAU.

Test for virucidal effect: Trichothecin (5.0 mcg/ml) was added to NDV suspension and residual infectivity was followed during incubation at 38.5°C as described previously[2)].

Effect on viral adsorption to host cells: NDV and CEF suspensions were incubated at 5°C with occasional stirring and unadsorbed infectivity was titrated[2)].

Measurement of protein synthesis: CEF sheets in test tubes were infected with NDV at a multiplicity of 50 PFU/cell and incubated at 38.5°C. The medium was replaced with fresh medium free of lactalbumin hydrolysates at 4.5 hours after infection and trichothecin (3.0 mcg/ml) and ^{14}C-amino acids (0.1 μC/ml) were added at 5 hours. Time course of incorporation of the radioactivity into acid-insoluble fractions was examined by freezing-and-thawing the cells and medium three times in a dry ice-acetone bath and collecting precipitates insoluble in 10% trichloroacetic acid (TCA) on Millipore filters (Millipore Filter Co., Mass., U. S. A.).

Synthesis of actinomycin D-insensitive RNA and analysis on methylated albumin-kiselguhr (MAK) column: The effect of trichothecin on viral RNA synthesis was examined in the presence of actinomycin D (2.0 mcg/ml) which inhibited the synthesis of cellular nucleic acids and unmasked viral RNA synthesis[3)]. NDV-infected cells were treated with trichothecin (3.0 mcg/ml) and actinomycin D for 1 hour (4~5 hours after infection) and were refed with fresh medium containing the antibiotics and ^{32}P-phosphate (50 μC/ml). Nucleic acids were extracted by the SDS (sodium dodecylsulfate)-phenol method and analysed by column chromatography[4)] on a methylated albumin-kieselguhr (MAK).

Measurement of viral growth: Multiplication of NDV was determined by titrating PFU and/or HAU after releasing cell associated viruses by three cycles of freezing-and-thawing in a dry ice-acetone bath[2)].

Chemicals used: Trichothecin was prepared from a mycelial acetone extract of *Trichothecium roseum* Link IFO 5772 according to the method described previously[1)]. Actinomycin D was a gift from Merck, Sharp and Dohme International, N. Y., U.S.A. Bovine serum albumin and SDS were purchased from Armour Pharmaceutical Co., Ill., U. S. A. and Koso Chemical Co., Tokyo, Japan, respectively. ^{14}C-Amino acids mixtures and carrier-free ^{32}P-phosphate were obtained from the Radiochemical Centre, Amersham, England.

Results

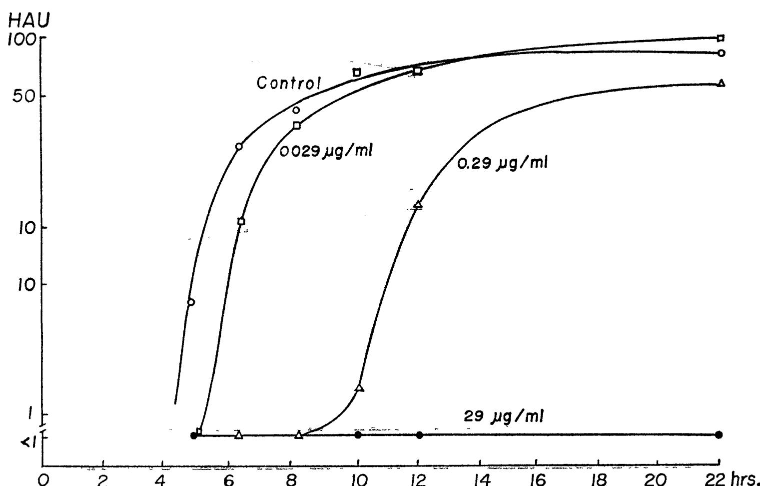
Dose Response of the Inhibitory Activity of Trichothecin on NDV Growth

When trichothecin was added at the time of infection (multiplicity of 50 PFU/cell), NDV multiplication in primary cultures of CEF was completely suppressed at a concentration of 2.9 mcg/ml. Addition of 0.29 mcg/ml of trichothecin lengthened the lag time before onset of hemagglutinin (HA) synthesis up to 10 hours of about double that of control (Fig. 1). Once the synthesis of HA began in the presence of 0.29 mcg/ml trichothecin, no difference was detected in any event examined such as rate and final yield of HA production. At such sub-inhibitory concentrations, HAU at 22 hours after infection did not differ from that of mock-treated controls, but direct microscopic observation revealed that complete destruction of the cell sheets was prevented as compared with the controls.

To confirm the multiplication of infective virus in the presence of trichothecin, the correlation between HAU and PFU was examined 17 hours after infection (Table 1).

Fig. 1. Dose-response of the inhibitory effect of trichothecin on the one-step growth of NDV.

After a 2-hour adsorption period at 5°C, infected monolayer cultures of CEF in test tubes were refed with fresh medium containing the antibiotic at the concentrations indicated. Viral growth was followed by HAU titration with duplicate tubes at each point.



The degree of inhibition of infective virus production and HA synthesis were nearly the same, and no accumulation of non-infectious virus was observed.

Table 1. Production of infective virus and HA in the presence of trichothecin

Concentration (mcg/ml)	% PFU	% HAU
0	100	100
3.2	<1.0	<1.0
1.6	<1.0	<1.0
0.8	10.0	21.3
0.4	13.8	25.0
0.2	31.3	42.5
0.1	78.8	85.0

Confluent monolayers of CEF were infected (50 PFU/cell) and allowed to stand at 5°C for 2 hours. Prewarmed medium containing trichothecin at the concentrations indicated was added after removal of unadsorbed viruses. PFU and HAU were titrated at 17 hours after the infection and were expressed in per cent of those of the controls.

Effect of Time of Addition on NDV Growth

Trichothecin could exert its antiviral activity whenever it was added during the one-step viral growth cycle (Fig. 2), and the complete inhibition of HA synthesis occurred 1.5 hours after its addition. During this lag period the rate of HA synthesis equaled that in controls and was then followed by an abrupt cessation.

Reversibility of Inhibitory Action of Trichothecin

Trichothecin (5.0 mcg/ml) was added to virus-infected cells at 0 time and removed after various periods of treatment to examine recovery of HA production from the inhibition by trichothecin. As shown in Fig. 3, HA synthesis was restored after removal of the antibiotic with cells pretreated up to 7 hours. The inhibitory action of the antibiotic was reversible even when infected cells were treated for 4 hours after infection (between 1.5～5.5 or 6.0～10.0 hours) in partially synchronized viral growth (Fig. 4). A lag period of 4～5 hours was observed in all of these cases.

Action of Trichothecin on Free NDV Particles and on Adsorption onto CEP

Trichothecin had no virucidal effect on free NDV particles at 38.5°C at higher concentrations (upto 10 mcg/ml) than required for complete suppression of vial growth.

Fig. 2. Effect of time of addition on the inhibitory activity of trichothecin (5.9 mcg/ml) on the growth of NDV.

CEF monolayer cultures in test tubes were fed with prewarmed medium after a 2-hour viral adsorption period at 5°C and were incubated at 38.5°C. At the times indicated trichothecin was added and viral growth in duplicate tubes was followed by HAU titration.

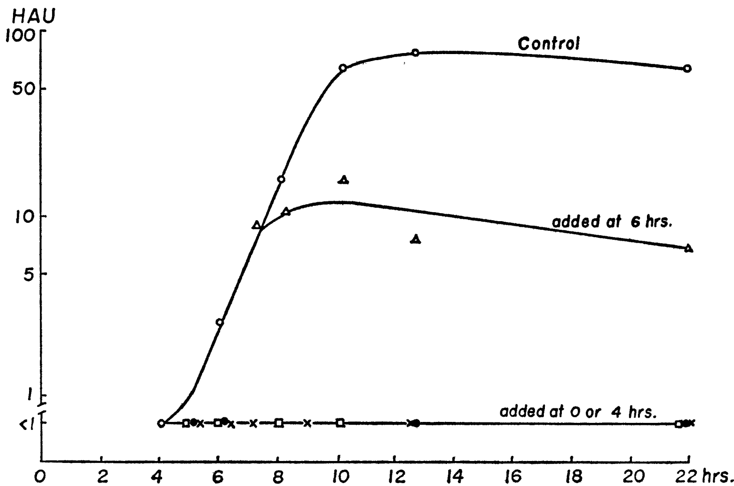
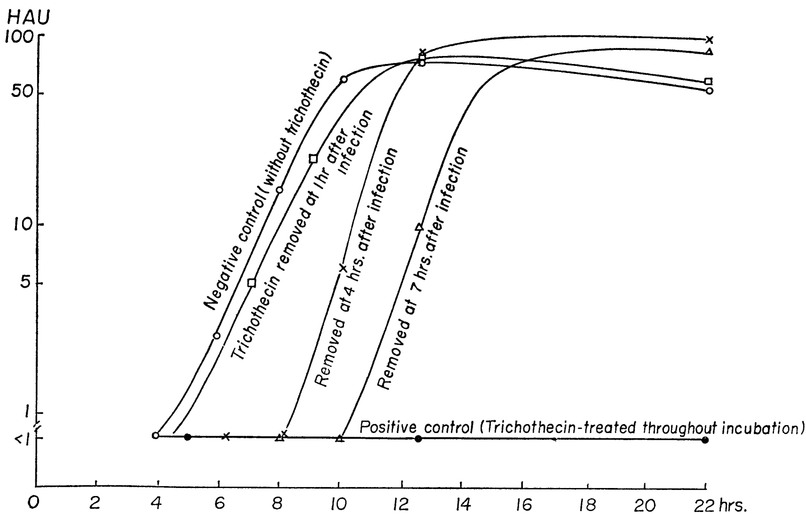


Fig. 3. Reversibility of the inhibitory effect of trichothecin on the multiplication of NDV with drug added at 0 time.

Trichothecin (5.0 mcg/ml) was added at 0 time and removed at the times indicated by washing the cell sheets three times. NDV growth was followed by HAU titration.



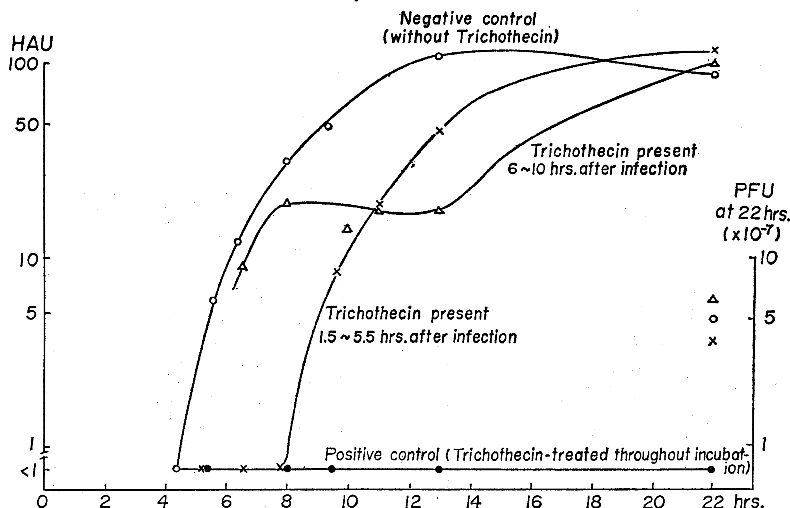
Adsorption of NDV to host cells was not affected by the presence of trichothecin (10 mcg/ml), and the rate of decrease of residual infectivity in the supernatant, after centrifugation, was the same in the presence and absence of the antibiotic.

Inhibition of Protein Synthesis by Trichothecin

In the above experiments it was found that trichothecin had no virucidal effect, did not affect viral adsorption to host cells and could exert its antiviral activity

Fig. 4. Effect of adding trichothecin (5.0 mcg/ml) after infection on the reversibility of the inhibition of viral growth.

Trichothecin was added to NDV-infected cell cultures after infection and removed after a 4-hour treatment period. Viral growth was followed by HAU titration and was checked by PFU titration at 22 hours after infection.

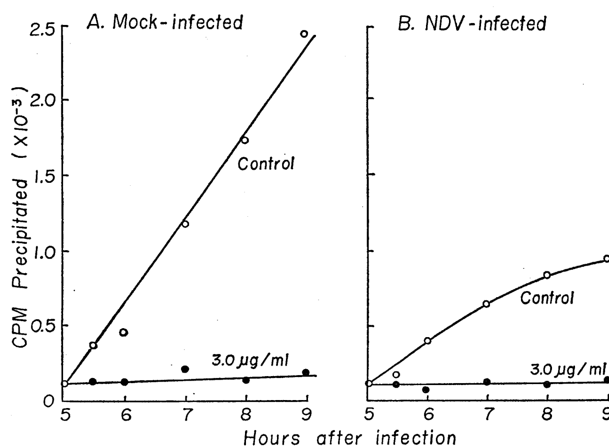


whenever it was added during a viral growth, and a good correlation was noted between infectious virus growth and HA production. These results indicated that trichothecin displayed its activity after viral adsorption and penetration and before maturation, thus the effects on macromolecular synthesis in virus-infected cells were examined.

Trichothecin (3.0 mcg/ml) suppressed completely and promptly after its addition the incorporation of a ^{14}C-amino acid mixture into the acid-insoluble fraction in both NDV-infected and mock-infected cells (Fig. 5). Reduced incorporation of radioactivity was observed with virus-infected cells in comparison with that in the control as by other workers[5,6,7,8].

Fig. 5. Effect of trichothecin (3.0 mcg/ml) on protein synthesis in NDV-infected and mock-infected cells.

Confluent monolayers of CEF in test tubes were infected or false-infected with NDV (50 PFU/cell), and trichothecin and ^{14}C-amino acids mixture were added at 5 hours after infection. Incorporation of radioactivity into 10 % TCA-insoluble fraction was measured by collecting precipitates on Millipore filters.

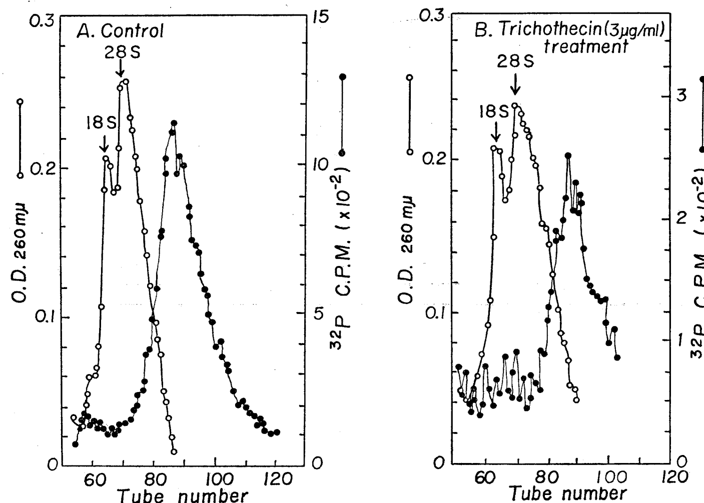


Effect on Actinomycin D-insensitive RNA Synthesis

Actinomycin D inhibits cellular RNA synthesis and unmasks viral RNA synthesis[3]. Employing this method, the effect of trichothecin on NDV-RNA synthesis was tested, and it was found that actinomycin D-insensitive RNA synthesis proceeded in the presence of trichothecin (3.0 mcg/ml) but to a much lesser extent (about 90 %) than

Fig. 6. Effect of trichothecin (3.0 mcg/ml) on actinomycin D-insensitive RNA synthesis.

CEF infected with NDV (50 PFU/cell) were treated with actinomycin D (2.0 mcg/ml) and trichothecin for 1 hour (4~5 hours after infection). ^{32}P-Phosphate was added at 5 hours after infection, and new synthesis of RNA was pursued by labeling with ^{32}P in the presence of the antibiotics. At 10 hours after infection incorporation of radioactivity was stopped by adding SDS (1.0 %) and ethylenediamine-tetraacetic acid (10^{-2} M). Nucleic acids were extracted by SDS-phenol method and precipitated with two volumes of ethanol after addition of nucleic acids prepared from chick embryos as a precipitation carrier and an optical density marker. Collected precipitates were analysed on MAK column chromatography (0.4 M→1.4 M NaCl/350 ml, 3 sec/drop, 4 ml/tube).



in the control. The antibiotics were added at 4 hours after infection and total radioactivity incorporated into RNA fraction was counted. WILSON and LOGERFO[5] and SCHOLTISSEK and ROTT[6] observed the same effect with puromycin and *p*-fluorophenylalanine. But characteristics of the RNA newly synthesized in the presence of such inhibitors have not been examined. ^{32}P-Labeled nucleic acids synthesized in the presence of actinomycin D and trichothecin were extracted and analysed on MAK column chromatography (Fig. 6).

RNA prepared from purified NDV was mostly eluted after 28S ribosomal RNA added as an optical density marker, and viral RNA prepared from infected cells and medium was also eluted in this region (Fig. 6A). When infected cells were treated with trichothecin, the radioactivity eluted in this region was less than the control (Fig. 6B), although the total incorporation of radioactivity into the RNA fraction was nearly the same in both experiments. The residual radioactivity was found to be eluted with a lower salt concentration (*ca.* 0.4 M). This same effect was observed using xanthocillin X monomethylether[4], a newly isolated antiviral antibiotic[9].

Discussion

Trichothecin was found to be a potent inhibitor of protein synthesis (Fig. 5) and its action was reversible (Figs. 3 and 4). Inhibition of NDV growth may be a result of cessation of protein synthesis, but determination of the primary site of the action for trichothecin awaits for further studies including experiments with cell-free protein synthesizing system. Trichothecin suppressed both viral and cellular protein syntheses

(Fig. 5), and is thus non-specific in its action. Many antibiotics have been reported to be specific inhibitors of bacterial protein synthesis (see reviews by TANAKA[10,11]). Kanamycin, streptomycin, fusidic acid, helvolinic acid, lincomycin, bottromycin, mikamycin A, mikamycin B and chloramphenicol, which have no effect on animal cell protein synthesis, were also ineffective on NDV growth using the agar-diffusion plaque-inhibition method at concentrations up to 4 mg/ml. A mixture of mikamycin A and mikamycin B (85 : 15) displayed antiviral activity at 15 mcg/ml. Cycloheximide, puromycin and blasticidin S showed anti-NDV activity in this test[4].

Early proteins necessary for NDV-RNA synthesis are almost all synthesized up to 4 hours after infection, and viral RNA synthesis has been observed to proceed at the same rate in the control and in the presence of inhibitors of protein synthesis such as puromycin and *p*-fluorophenylalanine[5,6]. In the case of trichothecin, the same effect was observed on the total incorporation of radioactivity into the RNA fraction. MAK column chromatography revealed that the radioactivity eluted in the mature NDV-RNA region was less and its elution profile was more heterogeneous using infected cells treated with trichothecin than with the controls (Fig. 6). These results suggest the requirement for continuous protein synthesis for viral RNA synthesis even at a late phase of viral infection. The same results were obtained with xanthocillin X monomethylether[4].

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