TUNICAMYCIN, A NEW ANTIBIOTIC. II

SOME BIOLOGICAL PROPERTIES OF THE ANTIVIRAL ACTIVITY OF TUNICAMYCIN

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Tunicamycin showed antiviral activity in the agar diffusion, cytopathic effect suppression and plaque reduction tests against the multiplication of Newcastle disease virus (NDV) in cultured chick embryo fibroblasts. It also inhibited virus multiplication in embryonated eggs. The antiviral activity was observed whenever the antibiotic was added during a one-step growth cycle of NDV. Reversal of the antiviral activity of tunicamycin was observed after removal of the antibiotic, but treatment for more than 2 hours permitted only slight recovery of the virus growth. Static cells were insensitive to tunicamycin, but the antibiotic effected on growing cells. These effects will be correlated with the mode of action of the antibiotic.

Streptomyces lysosuperificus nov. sp. produces an antiviral antibiotic. The active principle has been isolated and found to be a new antibiotic, and was named tunicamycin, after its mode of action. Production, isolation, and physical, chemical and biological properties have been reported in a previous paper of this series[1].

Tunicamycin is active against animal and plant viruses including both RNA and DNA viruses. Gram-positive bacteria, yeasts and fungi were also sensitive to the antibiotic. Effects on viruses and bacteria of *Bacillus* species were more remarkable than on others at low concentrations (0.1~1.0 mcg/ml). Some biological characteristics of the antiviral activity of tunicamycin are presented in this paper and discussed in relation to its mode of action.

Materials and Methods

Virus, cell and medium: The MIYADERA strain of NDV was prepared from chorioallantoic fluid of infected embryonated eggs and used without further purification. Primary cultures of chick embryo fibroblasts (CEF) were carried out as reported previously[2]. GEY's salt solution supplemented with lactoalbumin hydrolysate, yeast extracts and calf serum was employed as the medium for cell culture and virus multiplication[2].

Quantitation of virus multiplication and virus particles: Infective virus was titrated by counting plaques on infected monolayer cultures of CEF and expressed as plaque-forming units (PFU). Hemagglutination units (HAU) were determined and expressed as described previously[2].

Tunicamycin: Tunicamycin was isolated according to the previously reported method[1], and the same lot of three-times recrystallized preparation was used throughout the experiments reported here.

Details of the methods will be described in the text or legends.

Results and Discussion

1. Antiviral Activity of Tunicamycin as Determined by Various Methods

(1) Agar-diffusion plaque-suppression test

Two-day cultures of CEF in Petri dishes were infected with NDV to form about 5,000 plaques per dish. Infected monolayer cultures were overlaid with the soft agar medium containing neutral red. Paper discs (8 mm in diameter, thin, Tokyo Roshi Co.) impregnated with the antibiotic solution in methanol were put on the hardened agar layers. After a 2-day incubation at 39°C in a humidified incubator, diameters of plaque-free antiviral zones and inside cytotoxic zones where no incorporation of neutral red was observed were measured.

Table 1 shows that the minimum inhibitory concentration of tunicamycin was about 0.5 mcg/ml, and cytotoxicity was not detected even at 12,000 mcg/ml, the highest concentration tested. Then the calculated chemotherapeutic index, *i.e.*, the ratio of the maximum concentration tolerated by the cultured cells to the minimum concentration required for the inhibition of the virus growth, was more than 24,000.

Table 1. Anti-NDV activity of tunicamycin as determined by the agar-diffusion plaque-inhibition method.

Concentration (mcg/ml)	AVZ (mm)
12,000	41
6,000	37
3.000	35
1,500	32
750	29
325	28
162	26
81	25
40	22
20	19
10	17
5	15
2.5	13
1.2	14
0.6	12
0.3	±

Monolayer cultures of CEF in Petri dishes were infected with NDV. Antiviral activity was determined by the method described in the text, and expressed by diameters of antiviral zones (AVZ).

(2) Plaque reduction test

Monolayer cultures of CEF in Petri dishes were infected with NDV to form about 300 plaques per plate. After a 2-hour period of adsorption at room temperature, soft agar medium containing neutral red and tunicamycin at designated concentrations was overlaid. Plaques were counted after a three-day incubation at 39°C. Four dishes were used for each drug concentration. The effect of tunicamycin on multiplication of NDV was determined by plaque formation following virus multiplication, and expressed as per cent of the controls.

Table 2. Anti-NDV activity of tunicamycin as determined by plaque reduction test.

Concentration (mcg/ml)	% PFU	Cytotoxicity
Control	100	
0.010	<1	—
0.005	<3	—
0.002	12	—
0.001	18	—

Monolayer cultures of CEF in Petri dishes were infected with NDV, and soft agar medium containing neutral red and tunicamycin was overlaid as described in the text. Four dishes were used at each drug concentration. Plaques were counted on the third day after the infection. Effect of tunicamycin on multiplication of NDV was determined by plaque formation following virus multiplication, and cytotoxic effect on the antibiotic was examined by incorporation of neutral red by cultured cells.

Almost complete inhibition was observed at drug concentrations 0.01~1,000 mcg/ml. The effect of tunicamycin on virus multiplication decreased as the drug concentration was diluted, and all-or-none effect was not observed.

Toxicity of tunicamycin was not detected at the highest concentration examined during the three-day incubation.

(3) Suppression of cytopathic effect of NDV

Cell sheets formed in test tubes were infected with NDV at an input multiplicity of 10 PFU/cell. After a 2-hour period of adsorption, unadsorbed virus was removed by washing the cell layers and fresh medium containing tunicamycin was fed to the infected cells. Duplicate tubes at each drug concentration were incubated at 39°C. The cytopathic effect following virus multiplication was measured by a direct microscopic observation, and virus yields were quantitated by HAU titration.

Table 3. Anti-NDV activity of tunicamycin as determined by tube assay method.

Concentration (mcg/ml)	Cytopathic effect			HAU at 24 hours	
	24 hours	48 hours	72 hours	HAU	% inhibition
Control	++++ ++++	++++ +++	++++ ++++	320	
10	− −	− −	− −	<1	~100
2.0	− −	− −	− −	<1	~100
0.40	− −	− −	− −	3	99
0.080	− −	− −	+ −	72	77
0.016	− −	− +	++ ++	100	69

Cell sheets in test tubes were infected with NDV at an input multiplicity of 10 PFU/cell. Cytopathic effect was observed microscopically and expressed as follows ; − : without cytopathic effect, + : slight cytopathic effect, ++ : moderate cytopathic effect, +++ : severe cytopathic effect but without destruction of cell sheet, and ++++ : severe cytopathic effect with partial or complete destruction of cell sheets. Virus production was determined at the same time by HAU titration.

Table 3 shows that tunicamycin completely suppressed the cytopathic effect of NDV at 0.05 mcg/ml, and a delay in its occurrence was observed at lower concentrations. The result of HAU titration nearly paralleled the microscopic observation. About 20- to 50-fold concentration of tunicamycin was required for complete suppression of the virus multiplication in comparison with the results of plaque reduction test because of higher input multiplicity of infection in the former case.

Cytotoxic effect of tunicamycin was not observed at any drug concentration tested.

(4) Antiviral activity *in ovo*

Nine-day-old embryonated eggs were inoculated with NDV (10^3 PFU/egg), and tunicamycin was added simultaneously. The effect of the antibiotic on the virus multiplication in eggs was measured by degree of suppression of lethal effect of NDV on embryos (Fig. 1) or by titration of HAU in chorioallantoic fluids (Table 4).

Tunicamycin suppressed NDV multiplication when virus production was determined on the second day after the infection (Table 4), but only slight extension of survival of the NDV-infected embryos was observed, and no egg hatched.

In the above-described agar diffusion (Table 1), plaque reduction (Table 2) and suppression of cytopathic effect (Table 3) tests, tunicamycin had no cytotoxic effect on cultured cells at the highest concentrations examined. The antibiotic was toxic to embryos, however, no hatching was observed with eggs treated with tunicamycin. This difference in toxicity may be a reflection of difference of the action of tunicamycin on static cells and on growing cells.

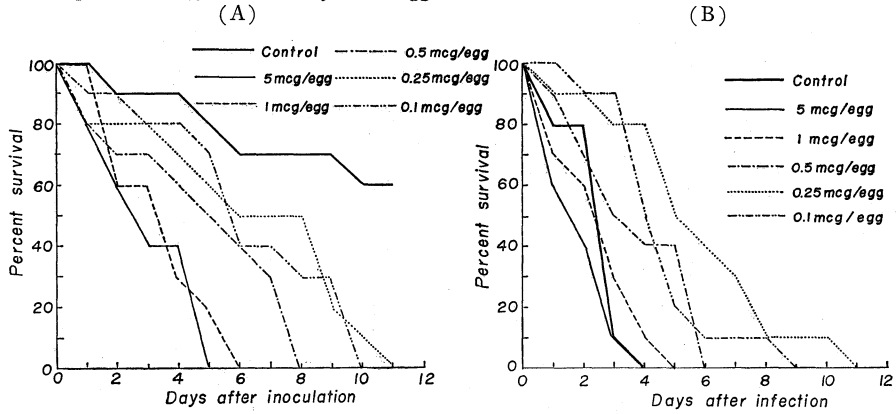
Table 4. Effect of tunicamycin on NDV growth in embryonated eggs.

Concentration (mcg/ml)	HAU on the second day
Control	1,600~3,200
5.0	<1
2.5	<1
1.0	<1~20
0.50	<1~20
0.25	<1~40
0.10	<1~160

Embryonated eggs were infected with NDV (10^3 PFU/egg) and tunicamycin was added at the same time. After incubation at 39°C for two days, chorioallantoic fluids were sampled and NDV in them was titrated and expressed as HAU.

Fig. 1. Effect of tunicamycin on growth of chick embryo and multiplication of NDV *in ovo*.

Nine-day-old embryonated eggs were inoculated with tunicamycin (A) or tunicamycin and NDV (B). Effect of tunicamycin on the growth of embryos and on NDV was determined by survival of treated eggs, experessed as %. Ten embryonated eggs were used for each determination.

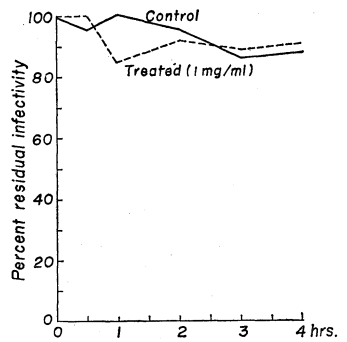


2. Effect of Tunicamycin on Free Virus Particles

NDV suspended in the medium was treated with tunicamycin at 39°C. Residual PFU were titrated with an appropriate dilution to dilute out the effect of the antibiotic at designated time intervals. As shown in Fig. 2, tunicamycin had no effect on virus infectivity under this condition of treatment.

Fig. 2. Effect of tunicamycin on NDV infectivity.

Suspension of NDV in the medium containing tunicamycin (1.0 mg/ml) was incubated at 39℃. At designated time intervals, infectivity of NDV was determined on monolayer cultures of CEF. Four cultures were used at each determination. Residual infectivity was expressed as % of input infectivity.



3. Effect on Virus Adsorption to Host Cells

Virus and cell suspensions in the medium containing tunicamycin were incubated at 39°C with occasional stirring, and cells and adsorbed virus were removed by centrifugation. Residual HAU in the supernatant were titrated.

Table 5 shows no difference between the controls and the treatments. The possibility of inhibition of virus adsorption to host cells by tunicamycin is eliminated.

Table 5. Virus adsorption onto CEF in the presence of tunicamycin.

Concentration (mcg/ml)	% Residual HAU
Control	13.5
100	9.8
10	15.2
1.0	14.0
0.1	8.8

Freshly prepared CEF were suspended in the medium and incubated at 39℃ with occasional stirring in the presence or absence of tunicamycin. After removal of adsorbed viruses by centrifugation, residual NDV in the supernatant was determined by HAU titration and expressed as % of input HAU.

4. Effect of Time of Addition on the Antiviral Activity

From the above-described experiments, tunicamycin seemed to exert its antiviral activity on NDV after virus adsorption onto host cells. When the antibiotic was added after virus adsorption, and the minimum inhibitory concentration of tunicamycin (0.5 mcg/ml) was employed to minimize secondary effects of the antibiotic, tunicamycin inhibited NDV

multiplication whenever it was added during the viral one-step growth cycle (Fig. 3). Possibilities of inhibition of penetration or uncoating of NDV by tunicamycin may be neglected because the antibiotic suppressed virus multiplication even when it was added 8 hours after the infection, an exponentially growing time. A precise determination of site of inhibition waits further investigation. Studies of the effect of tunicamycin on viral macromolecular synthesis suggest that the antibiotic inhibits some late stage of virus muliplication, including synthesis of viral envelope (manuscript in preparation).

5. Reversibility of the Antiviral Activity

Infected cell cultures in test tubes (at an input multiplicity of 10 PFU/cell) were treated with tunicamycin (0.5 mcg/ml) for designated time intervals at 39°C, and the antibiotic was removed by washing the cell sheets with prewarmed medium. Recovery of the virus multiplication after the removal

Fig. 3. Effect of time of addition of tunicamycin on NDV growth.

Monolayer cultures of CEF in test tubes were infected with NDV at an input multiplicity of 10 PFU/cell. After a 2-hour period of adsorption at room temperature, unadsorbed virus was removed by washing the cell sheets. Infected cell cultures were re-fed with prewarmed fresh medium and incubated at 39℃ (0 time). Tunicamycin (0.5 mcg/ml) was added at various times during the one-step growth cycle of NDV. Virus growth was followed by HAU titration.

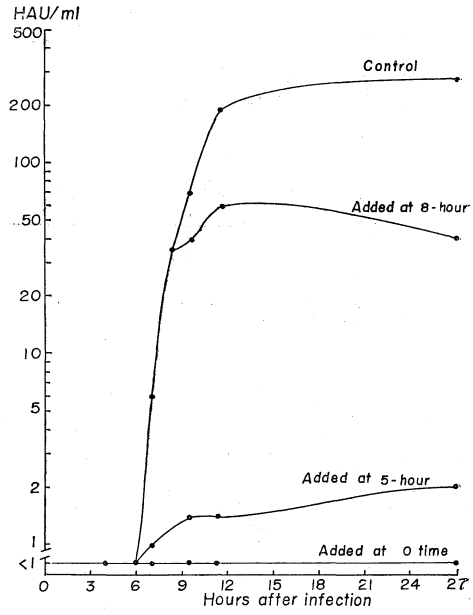
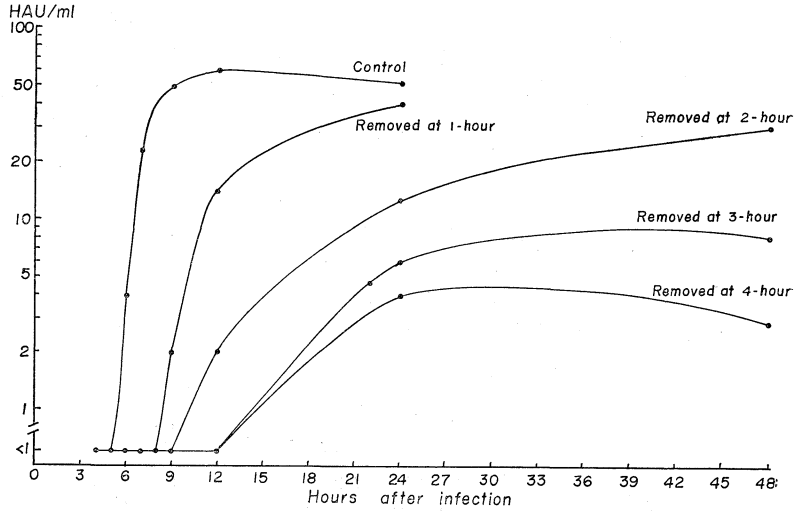


Fig. 4. Reversibility of the antiviral activity of tunicamycin after removal of the antibiotic by washing.

Cultures of CEF in test tubes were infected with NDV, and tunicamycin was added after a 2-hour period of adsorption. Infected cells were incubated at 39℃ (0 time), and tunicamycin was removed after various times of treatment. Virus multiplication was followed by HAU titration as described in the text.



was followed by HAU titration.

The inhibitory action of tunicamycin was fully reversed after the removal of the antibiotic with a few hours delay in beginning of titrable hemagglutinin synthesis when the duration of treatment with the antibiotic was less than 2 hours (Fig. 4). When the infected cells were incubated at 39°C with the antibiotic, HAU at 24 and 48 hours after the infection decreased as the period of treatment expanded. In particular, some drastic change had occurred between 2 and 3 hours after the treatment. The recovery of NDV growth as measured by HAU titration was very slight.

Table 6. Effect of pretreatment with tunicamycin on CEF growth.

	Duration of pretreatment (hours)	Cell growth after cell plantation			NDV growth (% HAU)
		24 hours	48 hours	72 hours	
Mock-treatment	1	++++ ++++	++++ ++++	++++ ++++	100
	2	++++ ++++	++++ ++++	++++ ++++	100
	3	++++ ++++	++++ ++++	++++ ++++	100
	4	++++ ++++	++++ ++++	++++ ++++	100
Treatment with tunicamycin (1.0 mcg/ml)	1	++++ ++++	++++ ++++	++++ ++++	100
	2	++++ ++++	++++ ++++	++++ ++++	100
	3	++++ ++++	++++ ++++	++++ ++++	100
	4	++ ++	+++ +++	++++ ++++	100

Monodispersed CEF in the medium was treated with tunicamycin (1.0 mcg/ml) at 39°C. At designated time intervals, an aliquot of suspension was sampled, and cells were collected by centrifugation. They were washed once with the medium and inoculated in test tubes. Cell growth was determined by microscopic observation and expressed as the degree of confluency of cell sheets as follows; − : no attachment of cells to vessel wall, + : 1~25 % of cnfluency, ++ : 26~50 % of confluency, +++ : 51~75 % of confluency, and ++++ : 76~100 % of confluency. On the fourth day of cultivation of pretreated cells, cell sheets formed in test tubes were infected with NDV at an input multiplicity of 10 PFU/cell. After a 24-hour period of incubation at 39°C, virus production was determined by HAU titration and expressed as % of mock-treatment controls.

To answer whether the above change was cellular or viral, confluent monolayers were treated with tunicamycin for designated time intervals and infected with NDV after removal of the antibiotic. A similar change was observed in this case as shown in Fig. 5. This result may suggest that the very slight reversal of the action of tunicamycin on the virus multiplication after a prolonged duration of treatment of infected cells is caused by some changes in the host cells. This effect on host cells is not cidal because treatment of freshly prepared monodispersed cells for 4 hours before inoculation only slightly affected the cell growth (Table 6). NDV could multiply well in monolayer cultures of these cells pretreated with tunicamycin. Virus infecton was carried out with confluent cell sheets and cell growth was not so prominent as in the case where the effect on cell growth was tested. Actively growing cells may be able to recover from inhibition by tunicamycin after the removal of the antibiotic, but cells in the state of confluency may be unable to activate their unknown mechanism of recovery. The change in the reversibility of NDV multiplication may be related to membrane synthesis (maunscript in preparation).

6. Effect on Cell Growth

Monodispersed cells prepared from chick embryos were distributed evenly into test tubes. Tunicamycin was added at the same time or at 12 hours after the cell inoculation. Cell growth was followed by microscopic observation and was expressed

Fig. 5. Effect of treatment of cell cutures of CEF with tunicamycin before NDV infection.

Tunicamycin (0.5 mcg/ml) was added to monolayer cultures of CEF in test tubes, and the cells were incubated at 39℃. Tunicamycin was removed at the time of infection (10 PFU/cell) by washing the cell sheets. After a 2-hour period of adsorption at room temperature, infected cell cultures were re-fed with fresh medium and incubated at 39℃ (0 time). Virus multiplication was followed by HAU titration.

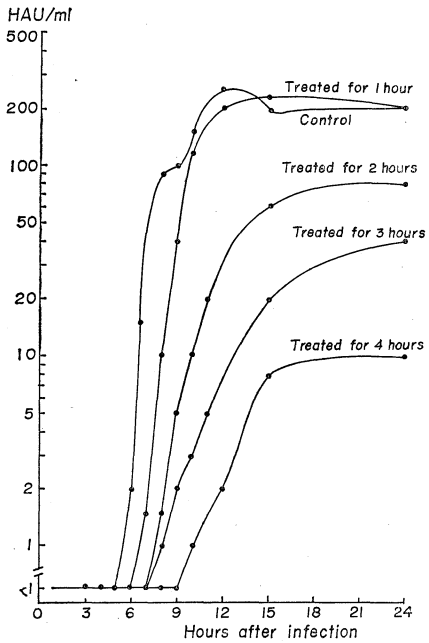


Table 7. Growth of CEF in the presance of tunicamycin.

Concentration (mcg/ml)	Cell growth			
	added at the time of cell inoculation		added after 12-hour incubation	
	at 24 hours	at 55 hours	at 24 hours	at 55 hours
10	++ / ++	+ / +	+++ / +++	++ / ++
5.0	++ / ++	+ / +	+++ / +++	++ / ++
2.5	++ / +	+ / +	++++ / +++	+++ / ++
1.25	++ / +	+ / +	+++ / ++	++ / ++
0.63	+++ / ++++	++ / ++	++++ / ++++	+++ / +++
0.31	++++ / ++++	++ / +	++++ / ++++	++ / +++
0.16	++++ / ++++	++ / ++	++++ / ++++	+++ / ++++
0.08	++++ / ++++	+++ / +++	++++ / ++++	++++ / ++++
0.04	++++ / ++++	++++ / ++++	++++ / ++++	++++ / ++++
0.02	++++ / ++++	++++ / ++++	++++ / ++++	++++ / ++++
Control	++++ / ++++	++++ / ++++	++++ / ++++	++++ / ++++

Freshly prepared CEF were suspended in the medium and distributed evenly into test tubes. Cell cultivation was carried out at 39℃. Tunicamycin was added at the time of cell inoculation or after a 12-hour period of incubation. Cell growth was observed microscopically and expressed as described in the legend of Table 6.

as described in the legend (Table 7).

When tunicamycin was added at the time of cell inoculation, growth inhibition was more pronounced than when added at 12 hours, after cells had attached to the vessel walls and cell division had begun to some extent.

When monodispersed cells were treated with tunicamycin and the antibiotic was removed at the designated times, the inhibiting effect on cell growth was reversed as described before (Table 6).

Studies on the mode of action of tunicamycin on cell and virus multiplication suggest that the antibiotic interferes with membrane synthesis, especially glycoproteins. Wheat-germ phytoagglutinin agglutinates cells transformed by oncogenic viruses[3]. This phenomenon is attributed to incompleteness of the carbohydrate chain of membrane glycolipids[4]. N-Acetylglucosamine has been shown to act as a receptor for the phytoagglutinin[5]. The antiviral activity of tunicamycin was partially reversed by some N-acetyl-aminosugars[6] and the antibiotic interferes with sugar metabolism. Thus it may be interesting to investigate whether cells grown in the presence of the antibiotic can be agglutinated by such phytoagglutinins. A preliminary experiment shows that chick embryo cells treated with tunicamycin before confluency and grown

in the presence of the antibiotic can be agglutinated by a crude wheat germ lipase to more extent than the controls (unpublised data).

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