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Effects of Actinomycin on Growth and Hemagglutinin Production of Vaccinia Virus

YOSHIHISA FUJIO

Department of Biology, Faculty of Science, Osaka University, Osaka
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SUMMARY

The action of actinomycin, an inhibitor of RNA synthesis, on hemagglutinin production, B type inclusion body formation, and virus yield in L cells infected with vaccinia virus, was investigated. The following results were obtained.

1. The growth of vaccinia virus and hemagglutinin production were completely inhibited by the addition of actinomycin to virus-infected cells.

2. Concentrations of actinomycin which suppressed the growth of virus and hemagglutinin production permitted B type inclusion body formation.

3. Eight hours exposure of host cells to actinomycin before viral infection had a slight effect on hemagglutinin production but this treatment inhibited B type inclusion body formation and virus yield, suggesting that viral DNA synthesis may depend on host cell RNA synthesis and that hemagglutinin production may not depend on host cell RNA synthesis.

4. Actinomycin inhibited B type inclusion body formation, hemagglutinin production, and virus yield when added within 4, 8, and 20 hours respectively after viral infection.

INTRODUCTION

Actinomycin is known to be one of the polypeptide antibiotics which inhibit growth of mammalian cells and gram-positive bacteria. It has been reported that actinomycin inhibits DNA-dependent RNA synthesis of mammalian cells (Reich *et al.*, 1961) and the enzyme activity from these cells (Goldberg and Rabinowitz, 1962). Recently, Reich *et al.* (1962) reported that actinomycin inhibited growth of DNA viruses but not of RNA viruses infecting mammalian cells.

The inhibitory action of actinomycin on DNA-dependent RNA synthesis is due to its action in binding DNA which is a primer in RNA synthesis. This strong binding is maintained during ultracentrifugation (Rauen *et al.*, 1960), zone-electrophoresis (Kawamata and Imanishi, 1960), and dialysis (Kirk, 1960).

The present paper reports the action of actionomycin on hemagglutinin production and B type inclusion body formation which are useful indicators of viral protein and DNA syntheses respectively (Kato *et al.*, 1959, 1960). Results are given on the yield of viruses in L cells infected with vaccinia viruses, whose genetic material is DNA.

MATERIAL AND METHODS

Viruses

The vaccinia virus used in the present work was the HA+ substrain of the IHD strain described previously (Fujio, 1962). Twenty four hours after inoculation of viruses into L cells, the infected cells were frozen and thawed. After centrifugation at 3,000 rpm for 10 minutes, the supernatant was used as the viral sample. Approximately 1.5×10^5 virus particles per bottle were inoculated into L cells.

Infectivity titration

Infectivity was titrated in L cells by the 50 per cent endpoint method in tube titrations ($TCID^{50}$). Monolayer cultures containing 2.0×10^5 L cells were prepared in a series of 15×100 mm test tubes. The culture media were removed from the tubes and 0.1 ml of viral sample was added to the cells. Four test tubes were used for each dilution of viral sample. After the cells had been incubated at 37°C for 2 hours to permit adsorption of virus, 1.5 ml of medium were added to each tube. Cytopathic changes in monolayer cultures in the test tubes were observed macroscopically and microscopically through the glass wall. Doses producing fifty per cent cytopathic changes were calculated by Reed and Muench's method from the number of test tubes showing cytopathic changes during a 7-day observation period.

Tissue culture

Approximately 1.0×10^5 L cells were dispersed in medium in a square bottle containing a 10×30 mm coverslip. The medium was a mixture of YLH solution (consisting of Hank's balanced salt solution, 0.5 per cent lactalbumin hydrolyzate and 0.1 per cent yeast extract) with 5 per cent bovine serum. Infected cells were cultured in YLH solution containing one per cent calf serum.

Hemagglutinin titration and hemadsorption

Twofold serial dilutions of viral suspensions were prepared in phosphate buffered saline (PBS) at pH 7.2. To 0.1 ml of each dilution was added an equal volume of PBS containing a suspension of 2 per cent red blood cells of selected fowls. After shaking, the mixtures were kept at room temperature for one hour and their patterns were recorded. The hemagglutination titer was expressed as the reciprocal of the highest dilution showing complete agglutination. Titers were recorded as HA units per milliliter of the original suspension. Hemadsorption was carried out by the methods described previously (Fujio, 1962).

Actinomycin

The actinomycin used in the present work was crystalline actinomycin S (Daiichi Seiyaku Pharmaceutical Co., Ltd). Because of the water insolubility of this antibiotic, the stock solution (1.0 mg/ml) was prepared by dissolving 10.0 mg of the antibiotic in 1.0 ml of ethanol and diluting the solution with 9.0 ml of distilled water. It was then stored in a refrigerator.

RESULTS

1. *Effect of post-treatment with actinomycin on the multiplication of vaccinina virus*

After a viral adsorption period for 2 hours without actinomycin, the cultures were washed and media containing various concentrations of actinomycin were added. After a 22 hour incubation period, hemagglutinin production, B type inclusion body formation, and the yield of viruses were examined. The results

Table 1. Effect on Vaccinia Virus of Post-treatment with Actinomycin

Concentration of Actinomycin	HA titer/tube	B type inclusion body formation (%)	Virus yield/tube	
			Infectivity titer	%
0.0 μg/ml	40	93.6	1.5×10^7	100.0
0.1	20	99.8	7.5×10^5	5.0
0.5	$<10^{(+)*}$	91.0	7.5×10^5	5.0
1.0	<10	91.0	1.5×10^5	1.0
5.0	<10	57.2	3.4×10^4	0.23
10.0	<10	6.5	1.5×10^3	0.01

1.5×10^5 viruses were inoculated into each tube. Actinomycin was added 2 hours after viral infection and was present throughout the 22 hour period.
* Hemadsorption was seen.

of this experiment are shown in Table 1. Twenty four hours after viral infection, hemagglutinin titers, the proportion of cells having B type inclusion bodies, and the virus yield were 40 HA units, 93.6 per cent, and 1.5×10^7 $TCID_{50}$ respectively. Fig. 1 shows hemadsorption and B type inclusion body formation in cells infected with viruses. B type inclusion body formation was inhibited significantly by 10.0 μg/ml of actinomycin. The growth of the virus was also inhibited when actinomycin was added to the media. At a concentration of 1.0 μg/ml of actinomycin, the yield of viruses was reduced to one hundredth of that in the control. Hemagglutinin production was completely inhibited by 1.0 μg/ml of actinomycin (Fig. 2). It is interesting that B type inclusion body formation was more resistant to actinomycin than hemagglutinin production and virus yield.

2. *Effect of pre-treatment of actinomycin on multiplication of vaccinia virus*

The cells were exposed to actinomycin for 8 hours, washed twice, and incubated for a further 16 hours in medium without the antibiotic. The pretreated

Table 2. Effect on Vaccinia Virus of Pre-treatment with Actinomycin

Concentration of Actinomycin	HA titer/tube	B type inclusion body formation (%)	Virus yiedl/tube	
			Infectivity titer	%
0.0 μg/ml	40	91.0	5.2×10^6	100.0
0.1	40	83.2	5.2×10^5	10.0
0.5	40	65.0	7.5×10^4	1.4
1.0	20	45.5	9.9×10^3	0.2
5.0	20	9.1	9.9×10^3	0.2
10.0	10	2.6	7.5×10^2	0.01

6.6×10^4 viruses were inoculated into each tube. Cells were cultured with actinomycin for 8 hours and were maintained without the antibiotic for 16 hours before being infected cells. Observed 24 hours after virus infection.

cells were then infected with viruses. An 8-hour pulse of actinomycin to host cells had a slight effect on hemagglutinin production, but this treatment inhibited B type inclusion body formation and virus yield (Table 2). B type inclusion body formation was significantly inhibited by an 8-hour exposure of actinomycin at 5.0 μg/ml (Fig. 3). Pre-treatment of actinomycin resulted in inhibition of virus yield which increased in proportion to the concentration of antibiotic.

3. *Effect of the time of addition of actinomycin on the mode of inhibition*

At various intervals after viral infection, actinomycin was added to the medium at a final concentration of 10.0 μg/ml, which was sufficient to inhibit hemagglutinin production, B type inclusion body formation, and virus yield. The effect of actinomycin on the expression of these characters was examined 24 hours after viral infection.

As shown in Table 3, actinomycin inhibited B type inclusion body formation when added within 4 hours and hemagglutinin production when added within 8 hours after viral infection. These facts suggest that hemagglutinin production was inhibited longer than B type inclusion body formation and the expression of these characters may be initiated by the synthesis of new RNA after viral infection. Actinomycin inhibited the virus yield when added within 20 hours after viral infection. The exposure of free virus particles to a concentration of actinomycin sufficient to prevent virus multiplication (10.0 μg/ml) did not cause any inactivation of virus.

Table 3. Effect on Vaccinia Virus of Time of Addition of Actinomycin

Hour at which actinomycin was added (10.0μg/ml)	HA titer/tube	B type inclusion body formation (%)	Virus yield/tube	
			Infectivity titer	%
Control	40	85.2	1.5×10^{7}	100.0
2	<10	2.6	1.5×10^{4}	0.1
4	<10	42.6	1.5×10^{4}	0.1
6	<10	52.3	1.5×10^{4}	0.1
8	<10(+)*	73.1	4.5×10^{4}	0.3
10	10	80.8	7.5×10^{4}	0.5
12	10	82.8	4.5×10^{5}	3.0
14	20	—	1.5×10^{6}	10.0
16	20	—	3.0×10^{6}	20.0
18	40	—	3.0×10^{6}	20.0
20	40	—	1.5×10^{7}	100.0
22	40	—	2.5×10^{7}	152.0
24	40	—	2.5×10^{7}	152.0

1.5×10^{5} viruses were inoculated into each tube. Actinomycin was added at various intervals after viral infection.

* Hemadsorption was seen.

DISCUSSION

Reich *et al.* (1962) reported that actinomycin at appropriate concentrations selectively suppressed RNA synthesis in L cells. Concentrations of inhibitor which diminish RNA synthesis by 99 per cent permit substantial protein and DNA syntheses for prolonged periods. The results reported in this paper show that concentrations of actinomycin which completely inhibit hemagglutinin production and virus yield permit B type inclusion body formation, which is an indicator of viral DNA synthesis. This suggests that the action of actinomycin in decreasing RNA synthesis is related with hemagglutinin production and viral protein synthesis for maturation but not with viral DNA synthesis.

After 8 hours exposure to actinomycin B type inclusion body formation and virus yield in host cells was inhibited but hemagglutinin production was not inhibited completely. This suggests that viral DNA synthesis but not hemagglutinin production may depend on host cell RNA synthesis. Thus, this suggests that host cell RNA may play an important role in vaccinia virus multiplication.

Actinomycin inhibited B type inclusion body formation when added within 4 hours after viral infection and hemagglutinin production when added within 8 hours after viral infection. Thus in cells infected with the virus, new RNA which plays a specific role in viral DNA synthesis and hemagglutinin production may be synthesized, and actinomycin may affect this new RNA synthesis. Tamm *et al.* (1960) obtained evidence with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), of an inhibitor of RNA synthesis, and found that synthesis of new RNA was necessary for the replication of two DNA viruses, vaccinia and adenovirus. Ikegami *et al.* (1960) found that DRB and RNase were only effective when L cells were exposed to them before ectromelia virus infection, indicating that a metabolic disturbance in RNA in the host cells was enough to suppress viral DNA synthesis. Sheek and Magee (1961) demonstrated the formation of an RNA-containing body in the nucleus very soon after infection and suggested that such a nuclear area, rich in RNA, might migrate into the cytoplasm. They considered that the RNA-protein template established by the viral gene in such an area might direct the synthesis of enzymes for viral synthesis. It was also found with puromycin, an inhibitor of protein synthesis, that early protein synthesis was necessary for vaccinia viral DNA synthesis (Fujio, unpublished). Hanafusa (1961) has shown that DNA polymerase activity became maximal 6 hours after infection of cells with vaccinia virus.

When actinomycin was added 20 hours after viral infection, viral growth could not be prevented by the inhibitor. This seems to show that at this time the process of virus synthesis had reached a stage when it was no longer inhibited by actinomycin. It must be noted that exposure of free virus particles to a concentration of actinomycin sufficient to prevent virus multiplication (10.0 μg/ml) did

not cause any inactivation of virus. This suggests that the inhibitory action of actinomycin is exerted on some early steps in the maturation process.

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REFERENCES

Fujio, Y. (1962). Detection of hemagglutinin production of vaccinia virus by hemadsorption. *Biken J.* **5,** 109-111.

Goldberg, I. H. and Rabinowitz, M. (1962). Actinomycin D inhibition deoxyribonucleic acid-dependent synthesis of ribonucleic acid. *Science* **136,** 315-316.

Hanafusa, T. (1961). Enzymatic synthesis and breakdown of deoxyribonucleic acid by extracts of L cells infected with vaccinia virus. *Biken's J.* **4,** 97-110.

Ikegami, N., Kato, S. and Kamahora, J. (1960). Studies on the inhibitory activity of 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), ribonuclease and proflavine on the one-step growth cycle of mouse pox virus (ectromelia virus) in L cell tissue culture. *Biken's J.* **3,** 57-76.

Kato, S., Takahashi, M., Kameyama, S. and Kamahora, J. (1959). A study of the morphological and cyto-immunological relationship between the inclusions of variola, cowpox, rabbitpox, vaccinia (variola origin) and vaccinia IHD and a consideration of the term "Guarnieri body". *Biken's J.* **2,** 353-363.

Kato, S., Kameyama, S. and Kamahora, J. (1960). Autoradiography of cells infected with variola and cowpox viruses with H^3-thymidine. *Biken's J.* **3,** 183-189.

Kawamata, J. and Imanishi, M. (1960). Interaction of actinomycin with deoxyribonucleic acid. *Nature* **187,** 1112-1113.

Rauen, H. M., Kersten, H. and Kersten, W. (1960). Zur Wirkungsweise von Actinomycinen. *Hoppe-Seyler's Z. Physiol. Chem.* **321,** 139-147.

Reich, E., Franklin, R. M., Shatkin, A. J. and Tatum, E. L. (1961). Effect of actinomycin D on cellular nucleic acid synthesis and virus production. *Science* **134,** 556-557.

Reich, E., Franklin, R. M., Shatkin, A. J. and Tatum, E. L. (1962). Action of actinomycin D on animal cells and viruses. *Proc. Natl. Acad. Sci.* **48,** 1238-1245.

Sheek, M. R. and Magee, W. E. (1961). An autoradiographic study on the intracellular development of vaccinia virus. *Virology* **15,** 146-163.

Tamm, I., Nemes, M. M. and Osterhout, S. (1960). On the role of ribonucleic acid in animal virus synthesis. I. Studies with 5, 6-dichloro-1-β-D-ribofuranosylabenzimidazole. *J. Exptl. Med.* **111,** 339-349.

EXPLANATION OF PHOTOGRAPHS

Fig. 1. Hemadsorption of fowl red blood cells around L cells infected with HA+ vaccinia viruses. Giemsa staining. ×400

Hemadsorption and B type inclusion body formation are seen.

Fig. 2. Inhibition of hemadsorption by post-treatment of actinomycin around L cells infected with HA+ vaccinia viruses. Giemsa staining. ×400

Hemagglutinin production was completely inhibited but B type inclusion body formation was unaffected by 1.0 μg/ml of actinomycin.

Fig. 3. Hemadsorption around L cells infected with HA+ vaccinia virus after pre-treatment of actinomycin. Giemsa staining. ×400

Hemagglutinin production was unaffected but B type inclusion body formation was significantly inhibited by 8 hours exposure to 5.0 μg/ml of actinomycin before viral infection.

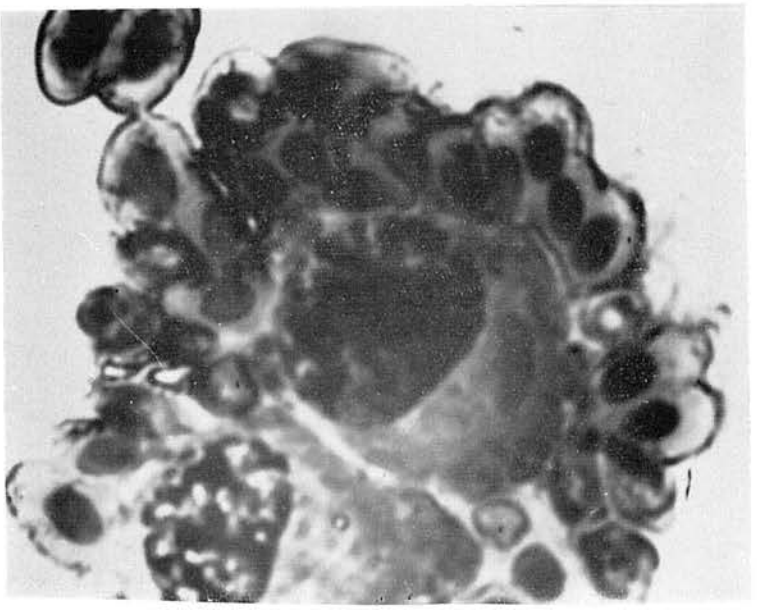

Fig. 1

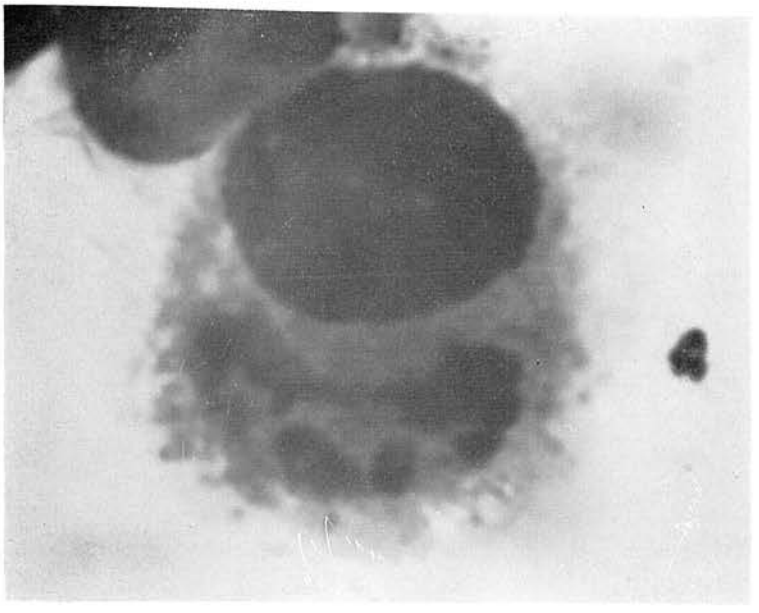

Fig. 2

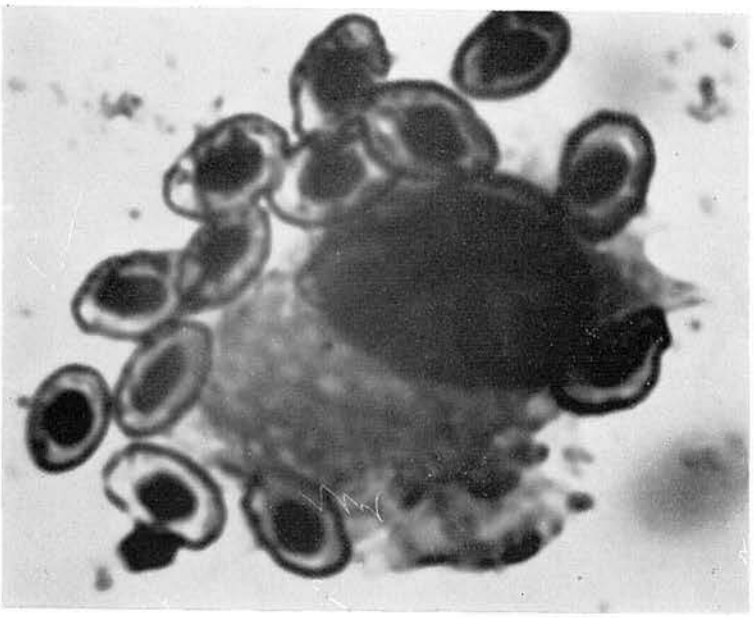

Fig. 3