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Inhibitory Action of 2-Mercapto-1-(β-4-pyridethyl)benzimidazole on Viral Neuraminidase Synthesis in HeLa Cells infected with Myxoviruses[1]

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Synthesis of viral-specific neuraminidase (NAase), one of the structural proteins of influenza virus, was inhibited by addition of 2-mercapto-1-(β-4-pyridethyl)benzimidazole (MPB), at a concentration of 50 μg/ml, to culture medium of infected HeLa cells during the first 1 hr post infection, and this inhibition was easily released by changing medium containing the drug to the drug free medium. Addition of MPB later than 5 hr, however, showed little inhibition of viral NAase induction in infected cells. Moreover, this drug as well as actinomycin D did not prevent the synthesis of NAase in infected HeLa cells with Newcastle disease virus. The mode of inhibitory action of MPB was very similar to that of actinomycin D excepting that MPB acted reversibly.

2-mercapto-1-(β-4-pyridethyl)benzimidazole (MPB) was reported as a reversible inhibitor of cellular ribonucleic acid (RNA) synthesis,[3] being used in several laboratories as an inhibitor of RNA synthesis.[4] However, the action mechanism of MPB has not been entirely understood yet. In a recent work, we have indicated that MPB blocked reversibly the nucleolar RNA synthesis of HeLa cells inhibiting through functional organizations of nucleoli according to biochemical, cytochemical and electronmicrographical investigations.[5] Based on these results, it was attempted to elucidate the mode of action of MPB on a replication process of two distinguished myxoviruses, influenza virus and Newcastle disease virus (NDV), because replication of the former virus is blocked by addition of actinomycin D but that of the latter is not.[6]

The present paper is concerned with an effect of MPB on the synthesis of neuraminidase (NAase), one of the structural proteins coded by the viral genome of influenza virus[7] and NDV,[6b] and a good marker of viral replication. It has been concluded that MPB, similar to actinomycin D, inhibits the replication of influenza virus but does not the replication of NDV.

Materials and Methods

Cells, Media and Virus——HeLa cells (given from Dr. Syverton) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum as a growth medium (GM). A maintenance medium (MM) for the experiment was prepared by adding 2% calf serum to MEM. The strain Aichi (A_2) of influenza virus was used throughout because this strain was able to multiply in HeLa cells almost the same to a proto-

1) Presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April 1974.
2) Location: *Shirokane, Minato-ku, Tokyo, 108, Japan.*
3) R.A. Bucknall and S.B. Carter, *Nature*, **213**, 1099 (1967); W.P. Summers and G.C. Mueller, *Biochem. Biophys. Res. Commun.*, **30**, 350 (1968).
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5) H. Nishigori and C. Nishimura, Abstracts of papers, the 47th Annual Meeting of the Japanese Biochemical Society, Okayama, October 1974.
6) *a*) R.D. Barry, D.R. Ives, and J.G. Cruickshank, *Nature*, **194**, 1139 (1962); *b*) D.W. Kingsbury, *Current Topics in Microbiology and Immunology*, **59**, 1 (1972).
7) W.G. Laver and E.D. Kilbourne, *Virology*, **30**, 493 (1966).

type strain (PR 8-R) of influenza virus.[8] The seed viruses for the experiments were prepared in a form of allantoic fluid of infected chick-embryo.

Inoculation of Influenza Virus and NDV to Cultured Cells——HeLa cells were seeded at a density of 5×10^5 cells per a plaque bottle and cultivated for 2 days at 35°. After removal of GM, the monolayers were washed once with Hanks-PBS solution and they were inoculated with 0.5 ml of the virus suspension at moi about 10. The inoculum virus was removed after 1 hr adsorption period at 35° and the monolayers were washed, covered with 2 ml of MM and incubated at 35° for the indicated time.

Preparation and Assay of NAase Activity of Infected Cells——HeLa cells infected with virus were collected at an appropriate time after infection and washed three times with phosphate buffered saline (PBS). They were suspended in 0.5 ml of double distilled water and disrupted by freeze-thaw cycles. An assay system contained 0.1 ml of the cell-lysate (80—100 μg as an amount of protein[9]), 0.05 ml of 2.5% fetuin[10] and 0.35 ml of 0.4 M phosphate buffer (pH 5.5) in a final volume of 0.5 ml. The reaction mixture was incubated at 37° for 2 hr and then the amount of liberated N-acetylneuraminic acid was determined by the method of Aminoff.[11] Through all experiments, the triplicate cultures were prepared and the data expressed usually as an average of NAase activities of these cultures.

Results

Effect of MPB on the Synthesis of Virus-specific NAase in HeLa Cells infected with Influenza Virus

The synthesis of virus-specific NAase was determined in HeLa cells infected with influenza virus. As shown in Fig. 1, it started within 4—5 hr after infection and afterwards increased linearly until 15 hr. Since no remarkable increase of virus-specific NAase was demonstrated

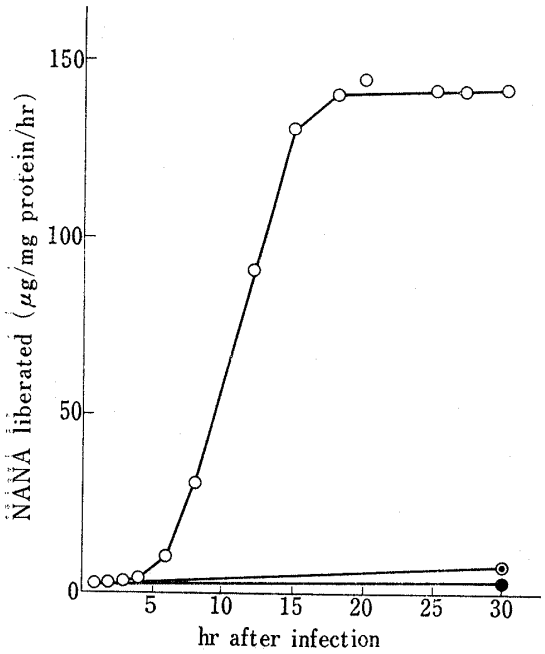

Fig. 1. Increase of Viral NAase in HeLa Cells infected with Influenza Virus

Influenza virus diluted with Hanks-PBS was irradiated with an ultraviolet lamp of National GL-15 from 20 cm height and inoculated to HeLa cells at moi about 10. NAase activity in cell-lysate was determined at 24 hr after infection and expressed as amount of liberated N-acetylneuraminic acid (NANA) (μg/mg protein/hr). ○—○: no irradiation with UV, ⊙—⊙: UV-irradiation for 1 min, ●—●: UV-irradiation for 3 min

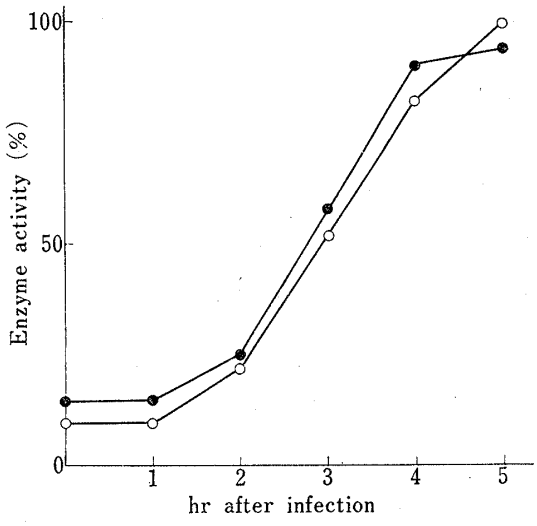

Fig. 2. Inhibition of Viral NAase Synthesis by MPB or Actinomycin D added at the Definite Intervals after Infection

MPB or actinomycin D was added to the infected cells at a concentration of 50 μg/ml or 0.5 μg/ml, respectively. MPB was dissolved in sterilized dimethylsulfoxide and 10 μl added per 1 ml of medium. For control dimethylsulfoxide was added. At 30 hr of infection, the activity of viral NAase was determined and data expressed as a percentage to the infected control. ○—○: MPB added, ●—●: actinomycin D added

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10) R.G. Ham and T.T. Puck, "Method in Enzymology," Vol. 5, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1965, p. 114.
11) D. Aminoff, *Virology*, **7**, 355 (1959).

in the cells infected with UV-inactivated influenza virus, newly synthesized NAase was elucidated as a protein coded by the genome of influenza virus.[7]

It was purposed to determine a drug-sensitive stage during the period of NAase synthesis in infected cells. The stage of drug sensitivity was evaluated by giving 50 μg/ml of MPB at definite intervals post infection and then the eventual activity of NAase in cells was determined at 30 hr post infection. These experiments complised a good measure of MPB sensitive stage during replication cycle of influenza virus. The similar experiment was carried out concerning the drug-sensitive stage of actinomycin D during NAase induction of influenza virus infected cells. Fig. 2 brings out the facts that the synthesis of viral NAase was almost inhibited either by addition of MPB or actinomycin D at the first 1 hr of infection, but partially inhibited by addition of the drug at 2 hr later. A less inhibition was observed by giving either MPB or actinomycin D later than 5 hr post infection. These results demonstrated that MPB and actinomycin D acted on the similar process in replicating cycle of influenza virus.

Reversible Inhibition of MPB to the Synthesis of Virus-specific NAase

Since the inhibitory effect of MPB on cellular RNA synthesis is known to be reversible,[3,5] we checked whether the inhibition of viral NAase synthesis by MPB could be reversible. By removing the drug at 5 hr post infection, the activity of NAase increased with a delay of 2.5 to 3 hr as shown in Fig. 3. Thus, the reversibility of MPB inhibition in synthesis of virus-specific NAase was proved. However, if actinomycin D was added immediately after removal of MPB at 5 hr of infection, no increase of viral NAase was observed. These results suggested that MPB prevented the actinomycin D-sensitive process. Inhibitory effect of actinomycin D on the synthesis of NAase could not be released even after removal of the drug.

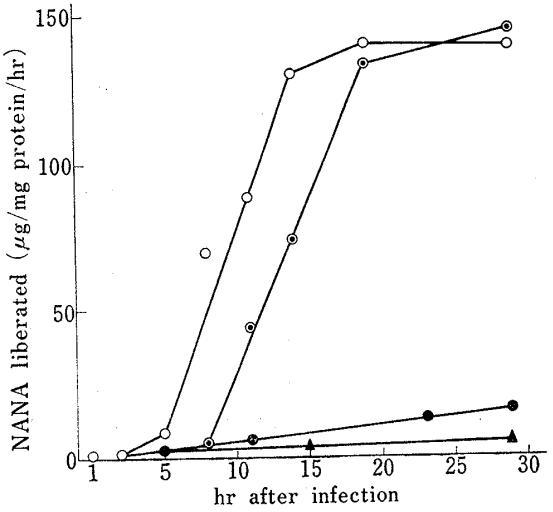

Fig. 3. Reversible Inhibition of MPB to Synthesis of Viral NAase in HeLa Cells

The activity of viral NAase was determined at the indicated time after infection and expressed as amount of liberated NANA (μg/mg protein/hr). For removing MPB, cells treated with the drug were washed three times with MM containing 1% dimethylsulfoxide and refeeded in fresh MM.
○—○: infected control, ●—●: with 50 μg/ml MPB continuously, ⊙—⊙: removed MPB at 5 hr after treatment, ▲—▲: 0.5 μg/ml actinomycin D added immediately after removal of MPB at 5 hr

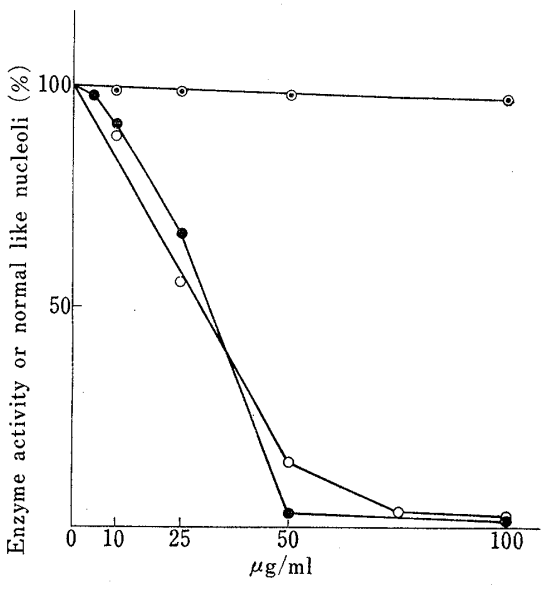

Fig. 4. Effect of MPB Concentration on Viral NAase Synthesis in HeLa Cells infected with NDV and Influenza Virus

Various amounts of MPB dissolved in dimethylsulfoxide were added to cell culture at the beginning of infection and yield of the enzyme at 30 hr post infection was determined. Data were expressed as a percentage to infected control with dimethylsulfoxide only. Nucleoli in MPB treated HeLa cells were examined by staining with pyronine G and methylgreen at 5 hr after addition of MPB. The percent of cells with normally staining and shape nucleoli was obtained by evaluating ten different areas on a slide under a light microscope.
○—○ : infected with influenza virus, ⊙—⊙ : infected with NDV at moi about 10, ●—●: percent of normal like nucleoli

Effect of MPB on the Synthesis of Virus-specific NAase in HeLa Cells Infected with NDV

The effects of various doses of MPB on the yield of NAase were examined in HeLa cells infected with either influenza virus or NDV. MPB was added simultaneously to the cells at the beginning of viral infection and kept remain during the period of infection. After the interval of 30 hr, the activity of NAase in the cells was determined. Since we demonstrated that MPB caused the fragmentation of nucleoli in HeLa cells,[5] the inhibitory effect of MPB on viral NAase synthesis was compared with the effect of the drug on the nucleolar disorganization. As shown in Fig. 4, dose-response of the two effects was almost coincided. Whereas, the synthesis of virus-specific NAase caused by NDV infection was unaffected by any doses of MPB, since replication of NDV does not always require nucleolar function of infected cells.[6b]

Discussion

Although it has been reported that MPB reversibly inhibits RNA synthesis in cultured mammalian cells while only minimally affecting protein synthesis,[3] Nakata and Bader[12] reported that the site of a primary action was not upon RNA synthesis since the drug inhibited nucleoside uptake into cells. Recently we have demonstrated that MPB, being different in its action mechanism from actinomycin D, prevented reversibly *de novo* nucleolar RNA synthesis according to biochemical and cytological investigations of HeLa cells.[5] In the present communication, it was shown that MPB had almost same action on RNA virus replication as actinomycin D. Namely, both drugs did not prevent NAase synthesis in NDV infected cells but that in influenza virus infected cells. This fact can be explained because of different replication mechanism of both viruses; NDV replicates only in cytoplasm which is distinguished from replication mechanism of influenza virus.[6b] According to studies on the replication mechanism of influenza virus by using actinomycin D, it was suggested that DNA-dependent step in nuclei was required at an early stage of virus development.[6a] However, as an alternative, it was reported that actinomycin D might break the conformation of invading viral RNA at the early stage of infection.[13] As discussed in the present paper, the synthesis of viral-specific NAase was inhibited by the addition of MPB, during the first 1 hr after infection, in the same way by actinomycin D. However, distinguished from the inhibition by actinomycin D, the effect of MPB on the replication was completely reversible by removal of the drug at 5 hr after MPB treatment. These observations indicated that the genome of influenza virus had remained intact in the infected cells during MPB treatment and that the early MPB-sensitive stage was needed for the replication of influenza virus. Moreover, it was noted that the induction period necessary for the synthesis of viral-NAase in MPB-treated cells was shortened when the drug was removed from the culture media, suggesting that a part of the initial events for protein biosynthesis had proceeded in the presence of MPB.

As shown in Fig. 4, the disorganization of nucleoli occurred differently by added MPB depending upon its doses and was closely related in parallel to the inhibition of viral-NAase synthesis in infected cells. From these facts, it is likely that nucleoli of infected cells are involved in the initiation of replicating cycle of influenza virus. In this connection, however, Mahy, *et al.*[14] reported a different view that DNA-RNA polymerase II of nucleoplasm shared an essential role for the replication of influenza virus. As the mechanism of influenza virus in mammalian cells has not yet fully explained, our experiments are progressing in a careful consideration to this point.

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