THE INHIBITORY EFFECT OF 4-AMINOPTERINE ON INFLUENZA VIRUS PROPAGATION IN TISSUE CULTURE

YOETSU HACHISUKA AND NOBUO KATO*

Department of Bacteriology, Nagoya University School of Medicine
(Director: Prof. Kazuo Ogasawara)

Some viruses propagate *in vitro* in a susceptible tissue maintained in a medium composed of inorganic salts and glucose. By introducing various metabolic antagonists in this host-virus system inhibition of viral propagation will occur. The inhibition may depend upon the competitive action with normal metabolites essential for enzymic processes in a host tissue which participate in viral propagation. Indeed, this technic has been used by several investigators and has proved to be a promising method for studying the nutritional requirements for viral propagation. Using this method, Ackermann has reported that *l*-methoxinine[1] which is an antagonist of *l*-methionine, malonic acid[2] and α-aminosulphonic acid[3] inhibit effectively the propagation of influenza virus without serious toxic effects on both host tissue and the virus itself. Cushing *et al.*[4] have reported the inhibitory effect of oxythiamine, an antagonist of thiamine, and desoxypyridoxine, an antagonist of pyridoxine.

In such a way the action of 4-aminopterine on the propagation of influenza virus in tissue culture has been studied. The results are reported in this paper.

MATERIALS AND METHODS

Virus and Tissue. The FM 1 strain of influenza type A′ virus was used. It had been passed in the chorioallantoic cavity of embryonate chicken egg. Allantoic fluid from infected embryonate chicken egg was used as the starting virus source. Tissue was the chorioallantoic membrane obtained from 10-14 day old embryonate chicken eggs.

Culture. The chorioallantoic membrane was washed with saline and finely minced with scissors. As a culture medium Tyrode solution was used. In the medium penicillin G (20 000 units/l) and streptomycin (50 mg/l) were added and phenol red (5 mg/l) was added to show directly the change of pH of the medium. Then pH of the medium was adjusted to 7.4 with sodium hydroxide or hydrochloric acid. 0.2 ml (200 mg) of the minced membrane and 2.0 ml of the medium were added into each Carrel's flask and then 0.1 ml of virus suspension was inoculated. The inoculum was prepared by making dilutions of infected allantoic fluid with saline solution to contain 8 hemagglutination titers in 0.1 ml.

Received for publication March 21, 1955.

* Present adress: The 1st Department of Internal Medicine, Nagoya University School of Medicine.

Solution of 4-aminopterine was added immediately in the first experiment or 3 hours after inoculation in the second experiment. In each flask the final volume of fluid and tissue was 3.0 ml. The cultures were incubated at 37° for 48 hours.

Virus titer. After incubation the medium was centrifuged and virus content in the supernatant was measured by hemagglutination titrations with chicken erythrocytes.

RESULTS

In the case of adding 4-aminopterine simultaneously with the inoculation of virus, its graded concentration demonstrated a proportional inhibition of the propagation of influenza virus (Table 1). Less than 100 γ/ml of 4-aminopterine had no effect. The presence of more than 400 γ/ml suppressed completely the viral propagation. In this case the change of pH of the medium was exactly the same as that of the medium containing no 4-aminopterine. From this it may be said that 4-aminopterine of these concentrations had no toxic effect on the activity of utilization of glucose of the host tissue.

TABLE 1. Inhibition of propagation of influenza virus (FM 1) in tissue culture (37°, 48 hours) by 4-aminopterine

*Concentration of 4-aminopterine (γ/ml)	Virus content (hemagglutination titer)	
	Before incubation	After incubation
0	<1	32
100	<1	32
200	<1	16
300	<1	4
400	<1	<1
500	<1	<1

* 4-aminopterine was added immediately after inoculation of the virus.

In the case of adding 4-aminopterine 3 hours after the inoculation the inhibition was the same (Table 2). From this it seems that the effect of 4-aminopterine was exerted on the intracellular virus multiplication rather than by blocking the attachment of the virus to host cells.

After contact of influenza virus with more than 500 γ/ml of 4-aminopterine for 48 hours death of the virus did not occur. It showed that 4-aminopterine has no virucidal effect at these concentrations.

TABLE 2. Inhibition of propagation of influenza virus (FM 1) in tissue culture (37°, 48 hours) by 4-aminopterine

*Concentration of 4-aminopterine (γ/ml)	Virus content (hemagglutination titer)	
	Before incubation	After incubation
0	<1	32
400	<1	<1

* 4-aminopterine was added 3 hours after the inoculation of the virus.

DISCUSSION

The data presented here prove the remarkable inhibitory effect of 4-aminopterine on the synthesis of influenza virus in tissue culture. In these experiments the interaction of 4-aminopterine and folic acid or its derivatives on the viral propagation was not seen. Hence, it was not clearly demonstrated whether the inhibiton was reversible and based on the competitive action between 4-aminopterine and folic acid or its derivatives. However, many investigators[5] have presented evidences that 4-aminopterine is a metabolic antagonist of folic acid or its derivatives. Folic acid has been proved to be connected with the synthesis of hypoxanthine[6] and thymine[7][8] both of which are constituents of nucleic acid. Knight[9] has clarified that nucleoprotein is an important fraction of influenza virus. It may be postulated that nucleic acid metabolism of the host tissue in which folic acid or its derivatives function is essential for the viral propagation.

SUMMARY

4-Aminopterine inhibits the propagation of influenza virus (FM 1) in tissue culture at concentrations shown to be without observable toxic effect on both the tissue and the virus itself. The possible mechanism of inhibition was discussed.

We wish to express our appreciation to Prof. Kazuo Ogasawara and Dr. Ikuya Nagata for their continued encouragement and guidance.

REFERENCES

1. ACKERMANN, W. W. *J. exp. Med.* **93**: 337, 1951.
2. ACKERMANN, W. W. *J. biol. Chem.* **137**: 421, 1951.
3. ACKERMANN, W. W. *Proc. Soc. exp. Biol. Med.* **80**: 362, 1952.
4. CUSHING, R. T. AND H. R. MORGAN. *Proc. Soc. exp. Biol. Med.* **19**: 497, 1952.
5. WOLLEY, P. W. *A Study of Antimetabolite.* New York: John Wiley and Sons, 1952.
6. SCHULMAN, M. P. *Fed. Proc.* **10**: 244, 1951.
7. FROMMEYER. *Am. J. med. Sci.* **213**: 135, 1947.
8. TOTTER. *J. Am. chem. Soc.* **73**: 1521, 1951.
9. KNIGHT, C. A. *J. exp. Med.* **85**: 99, 1947.