IN VITRO ACTIVITY OF N′,N′-ANHYDROBIS (β-HYDROXYETHYL) BIGUANIDE HYDROCHLORIDE AGAINST PARAINFLUENZA VIRUS TYPE 3

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INTRODUCTION

N′, N′-anhydrobis (β-hydroxyethyl) biguanide hydrochloride (ABOB) is an antiviral substance which has been used widely because of its prophylactic and therapeutic effect against influenza since MELANDER and others first reported its anti-influenza viral activity in 1960[1,2].

The Research Committee of Antiviral Substances in Japan (chairman : Prof. OSAMU KITAMOTO) conducted double blind test of this drug in the epidemic of influenza A2 and B[3,4], as well as in small epidemic of adenovirus (type 3) infection[5], and in the latter KITAMOTO *et al.* found the effectiveness of ABOB against adenovirus infection for the first time.

Later, KAJI *et al*[6] showed that this drug has inhibitory effect against adenovirus type 3 and 8 when tested *in vitro*.

There have also been many works concerning the efficacy of this agent against measles[7] or mumps[8].

In the following experiment, we studied the antiviral activity of ABOB against parainfluenza virus type 3 in cell culture system.

MATERIALS AND METHODS

virus

A newly isolated strain of parainfluenza virus type 3(hemadsorption virus type 1) was used in the study. This was passed in primary cynomolgus monkey kidney cell several times, and the stock virus fluid with infectivity titer of $10^{7.5}$ $TCID_{50}$/0.2 ml and hemagglutination titer of 1 : 128 was obtained. The stock virus was stored at −70°C until required.

cells

Primary cynomolgus monkey kidney cells (CMK) were purchased through a commercial source and were grown in the medium containing EARLE'S basal saline solution, lactalbumin hydrolysate (0.5 %), sodium bicarbonate (0.11%), inactivated bovine serum (2%), penicillin (200 units/ml) and streptomycin (200 mcg/ml). When 30×10^4 cells per a tube were seeded, confluent monolayers were usually formed in 5 days of incubation at 37°C.

HEp-2 cells were grown in EAGLE'S minimal essential medium, supplemented with 10% inactivated horse serum and containing 20 mg % sodium bicarbonate, 200 units/ml of penicillin and 200 mcg/ml of streptomycin.

Primary CMK were maintained with Medium 199 without serum, and HEp-2 cells with Medium 199 with 1% inactivated horse serum. These media contained 84 mg% sodium bicarbonate and appropriate quantity of antibiotics.

chemicals

Sterile 10 w/v% stock solution of ABOB was made up in double distilled water and stored at 4°C until required.

Toxicity of the agent against the cells was examined in the following way. ABOB was dissolved in various concentrations in maintenance medium and was administered to the monolayer cell sheet of primary CMK or HEp-2. The cell cultures were incubated at 37°C for 7 days changing the media every other day and the cytopathic changes were observed microscopically at the end of the incubation period. The result is shown in Table 1.

Table 1. Toxicity of ABOB against HEp-2 and MK cell sheet (incubation period: 7 days)

conc. of ABOB (mg/ml) \ cell	HEp-2	MK
10.0	D	D
5.0	D	D
2.5	N	D
1.0	N	N
0.5	N	N
0	N	N

D=damaged, N=not damaged

Primary CMK seemed to be more susceptible to the agent than HEp-2 cell. The concentration of ABOB used in the experiment was 0.5 mg/ml except in the isotope study, in which case it was 1.0 mg/ml.

Actinomycin D was a gift from the Department of Antibiotics, National Institute of Health of Japan. Five mg/ml of solution was prepared in pure ethyl alcohol and stored at −20°C.

Tritiated uridine (^{3}H labeled at 5 position, with specific activity of 5.0 Ci/mM) was purchased from Daiichi Chemicals Co., Ltd.

infectivity titration

Infectivity titration was performed by means of hemadsorption technique using guinea pig red blood cell suspension, because cytopathic changes produced by parainfluenza virus type 3 in either primary monkey kidney or HEp-2 cell are insufficient for quantitative assay of infectivity.

Serial ten-fold dilutions of the test virus were made in the maintenance medium and 0.2 ml of each dilution was inoculation to 2 tube cell cultures. Four fifth of a milliliter of maintenance medium was added and the cultures were incubated at 37°C for 5 days changing the medium every other day. At the end of the incubation, culture fluids were removed and the cell sheet was washed with phosphate buffered saline solution (PBS). One milliliter of 0.5% suspension of washed, citrated guinea pig red blood cells were added to each tube. Tubes were placed semihorizontally for 30 minuted at 4°C, the cultures were washed well with PBS and the tests were read microscopically at 10×10 magnifications. When more than 1/4 of the area showed hemadsorption, the culture was judged as positive.

hemagglutination test

Hemagglutination test was performed using 0.5% suspension of guinea pig red blood cell and at room temperature.

Serial two-fold dilutions of the test fluid were made in PBS and 0.4 ml of the red blood cell suspension were added to equal volume of each dilution. Hemagglutination pattern was read after 75 minutes and the reciprocal of the highest dilution that showed positive hemagglutination was taken as hemagglutination titer.

virus inoculation and drug administration

Administration of the drug to the cell sheet was done by changing the culture medium to the maintenance medium containing appropriate concentration of ABOB. The drug was usually administered 24 hours prior to virus inoculation and was present for the whole of the incubation period unless otherwise mentioned.

Virus inoculation was performed as follows. Monolayer cell sheet was washed with PBS, and 0.2 ml of the virus fluid was inoculated per a tube. After one hour's adsorption at 37°C, the cell sheet was washed well with PBS to remove the unadsorbed virus. The cultures were incubated with maintenance medium at 37°C, changing the medium every other day.

isotope study

When complete monolayer of HEp-2 cell was formed, with cell population of 25×10^4 per a tube, the cultured cells were treated with actinomycin D, 2 mcg/ml, for one hour and the RNA synthesis of the host cell was completely inhibited. In such a condition, parainfluenza virus can multiply within the cytoplasm of the host cell without being inhibited by this agent[9].

The virus at a multiplicity of 200 $TCID_{50}$/cell was then inoculated together with actinomycin. After one-hour adsorption time, the cultures were washed with PBS, and maintenance medium which contained actinomycin D and tritiated uridine at the concentration of 1 mcCi/ml was added. Unlabeled uridine at the concentration of 10 mcg/ml was administered at the same time.

Two tube cultures were removed immediately, 3,6 and 9 hours after the end of the adsorption time respectively and the samples were processed for counting trichloroacetic acid-precipitable radioactivity using liquid scintillation counter and the uptake of tritiated uridine by the actinomycin treated, parainfluenza virus inoculated HEp-2 cells was estimated.

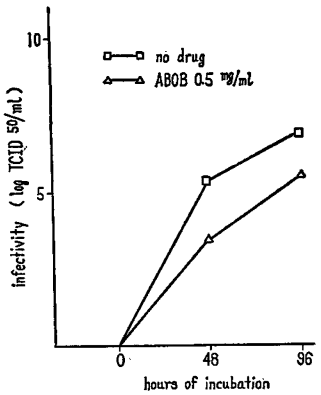
RESULTS

A. Effect of ABOB on production of infective parainfluenza virus in HEp-2 cell culture

When 0.5 mg/ml ABOB was administered 24 hours prior to virus inoculation and was present for the whole of the incubation period, virus yield at 48 and 96 hours following inoculation of $10^{3.0}$ $TCID_{50}$ remained $10^{3.5}$ and $10^{5.5}$ $TCID_{50}$/ml respectively, which infective virus titer at these points in the control cultures were $10^{5.3}$ and $10^{6.7}$ $TCID_{50}$/ml respectively, and at 48 hours there was 1.8 log unit's reduction in the production of infective virus and 1.2 log unit's reduction at 96 hours by 0.5 mg/ml of ABOB (Fig. 1).

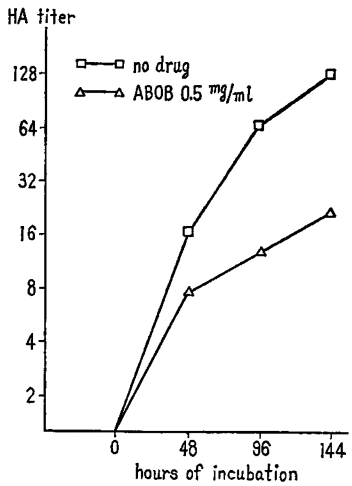
B. Effect of ABOB on free hemagglutinin pruduction by parainfluenza virus in primary CMK cell culture

Fig.1. Inhibitory effect of ABOB on parainfluenza virus type 3 multiplication in HEp-2 cells



Fluids from the primary CMK cell cultures inoculated with 10^3 $TCID_{50}$ of the virus were harvested at 48 hours' interval and their hemagglutinin titers were estimated. The result is shown in Fig. 2. If virus-cell system was incubated with

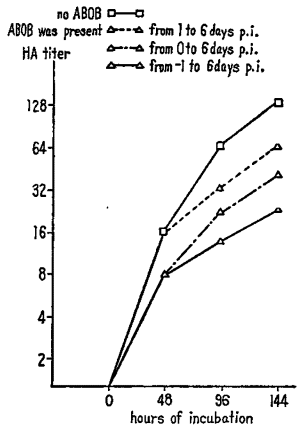
Fig.2. Inhibitory effect of ABOB on free hemagglutinin production by parainfluenza virus type 3 in primary monkey kidney cells



0.5 mg/ml of ABOB for 144 hours, titer of free hemagglutinin produced was around 1 : 16, while control showed as high as 1 : 128.

Inhibition of free hemagglutinin production was noticed even when the drug was administered at the same time with or even 24 hours after virus inoculation, if the culture systems were incubated with the drug for appropriately long period (Fig. 3).

Fig.3. Inhibitory effect of ABOB on free hemagglutinin production by parainfluenza virus type 3 in primary monkey kidney cells when the drug was administered variously during the incubation period

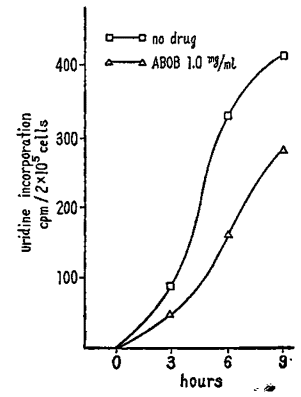


C. Effect of ABOB on tritiated uridine uptake by HEp-2 cells treated with actinomycin D and inoculated with parainfluenza virus

Tritiated uridine uptake by virus-host cell system when the cell was pretreated with 2 mcg/ml of actinomycin D increased gradually and reached the peak at 9 hours (Fig. 4. upper tracing).

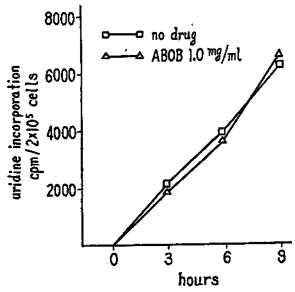
If the cells were treated with 1.0 mg/ml of ABOB for 24 hours before virus inoculation and then similarly processed with the control, uptake of uridine was moderately reduced (Fig. 4. lower tracing).

Fig.4. Incorporation of tritiated uridine into RNA of parainfluenza virus type 3 in HEp-2 cells



Uridine uptake by normal HEp-2 cells was never influenced by pre-treatment of the cells with 1.0 mg/ml of ABOB for 24 hours (Fig. 5).

Fig.5. Effect of ABOB on tritiated uridine incorporation into HEp-2 cells



DISCUSSION

ABOB in the concentration of 0.5 mg/ml inhibited the production both of infective virus and free hemagglutinin *in vitro* without damaging the host cell, although the degree of inhibition is not so great. Thus, there was 1 to 2 log unit's reduction in infective virus production and 1/4 to 1/8 reduction in free hemagglutinin production.

When RNA synthesis of the host cell is completely inhibited by actinomycin D, uptake of tritiated uridine by virus-host cell complex will reflect the degree of viral RNA synthesis within the cytoplasm because actinomycin D does not interfere with multiplication of parainfluenza viruses. Our experiment showed that 1.0 mg/ml of ABOB reduced the viral RNA synthesis by around 30%.

These results might make it probable, on condition that ABOB does not inactivate the virus outside the cell nor does it inhibit the virus adsorption to and penetration into the host cell[10], that ABOB primarily suppresses the synthesis of viral RNA within the host cell, and, as the result, there is a reduction in infective virus production or free hemagglutinin production as was observed in our experiment.

The exact mode of action of this agent, however, will remain to be resolved.

SUMMARY

Inhibitory activity of N′,N′-anhydrobis (β-hydroxyethyl) biguanide hydrochloride (ABOB) against parainfluenza virus type 3 was reported. When 0.5 mg/ml of ABOB was administered 24 hours prior to virus inoculation and was present for the whole of the incubation period, there was 1 to 2 log unit's reduction in infective virus production in HEp-2 cells and 1/4 to 1/8 reduction in free hemagglutinin production in primary cynomolgus monkey kindney cells.

ABOB in the concentration of 1.0 mcg/ml suppressed tritiated uridine incorporation into RNA to actinomycin D treated, parainfluenza virus infected HEp-2 cells by around 30%.

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