# EFFECT OF $N^1$,$N^1$-ANHYDROBIS-($\beta$-HYDROXYETHYL) BiGUANIDE HYDROCHLORIDE (ABOB) AGAINST ADENOVIRUS

MASARO KAJI, SEIJI KAMIYA, EIJI TATEWAKI, ZITSURO NAGAFUCHI and NOBORU FUJIWARA
The First Department of Internal Medicine, Faculty of Medicine
Kyushu University, Fukuoka, Japan

(Received April 9, 1965)

Many papers report the inhibitory effect of $N^1$,$N^1$-anhydrobis-($\beta$-hydroxyethyl) biguanide hydrochloride (ABOB) on influenza virus multiplication and its prophylactic and therapeutic evaluation in clinical trials. For the other viruses only few studies were made to test its antiviral activity. This paper reports the effect of ABOB against adenovirus multiplication in HeLa cells.

## Materials and Methods

**Virus** Adenovirus type 3, Koito strain* and type 8, Kanehisa strain[1] were used in the study. Both strains were propagated in HeLa cell cultures.

**Tissue Culture** HeLa cells were employed throughout this study. Method of culture and virus inoculation were previously described[2].

**Infectivity Titration** Specimens were made in serial tenfold dilution and 0.1 ml of each dilution was inoculated into a HeLa cell culture. Two culture tubes were used for each dilution. A tube was considered to be infected when at least 20 per cent of the cells showed viral cytopathic effect 12 days after inoculation. The 50 per cent endpoint was calculated by the REED and MUENCH's method[3].

**Complement Fixation Test** Titration of complement fixing (CF) titer of the fluid were carried out using rabbit antisera against adenovirus types 3 and 8. The fluid to be tested were heated at 56°C for 30 minutes and then clarified by centrifugation at 1,500 rpm for 10 minutes. 0.2 ml of serial 2-fold dilution of antigen, 0.2 ml of complement (2 units) and 0.2 ml of immune rabbit serum (4 units) were mixed and incubated at 37 °C for an hour. After the incubation 0.3 ml of 3 per cent sheep erythrocytes sensitized with an equal volume of hemolysin (2 units) was added and the mixture was incubated at 37 °C for 30 minutes before the tubes were read. The highest dilution of the fluid which resulted in a complete complement fixation was considered to the antigen titer.

* The strain was supplied by courtesy of Dr. H. FUKUMI, National Institute of Health, Tokyo.

** ABOB was kindly supplied from the Sumitomo Chemical Co.

**Hemagglutination Test of Adenovirus Type** Hemagglutinin (HA) titrations were performed at 37 °C by making serial two-fold virus dilutions in 0.5 ml to which 0.5 ml of 0.5 per cent suspension of human type 0 erythrocytes was added. After the mixtures were kept at 37 °C for 2 hours sedimentation patterns were read and reciplocal of the highest dilution which showed complete hemagglutination was taken as HA titer.

**ABOA** ABOB** was dissolved in distilled water to give the concentrations of 10 per cent and autoclaved at 1 lb for 20 minutes. Prior to the experiments ABOB solution was diluted to the appropriate concentrations with culture media.

**Eosin Exclusion Test** The test was used to examine the percentage of living cells in HeLa cell tubes with various concentrations of ABOB. Prior to dying, HeLa cells were digested with trypsin and dispersed by pipetting and the HeLa cell tube was placed vertically for a few minutes. When a majority of the dispersed cells sedimented spontaneously, one drop of medium with the sedimented cells was placed on a slide by a pipette. The small amount (approximately 0.01 ml) of 5 per cent eosin solution was added to the cells on the slide using a thin glass-bar and mixed with the cells sufficiently. When a cover-glass was placed on the mixture, the cells were ready to observe the percentage of viable cells. Because of selective permiability viable cells remained coloress whereas dead cells became diffusely red-stained.

## Results

**Viability of HeLa Cells Treated with ABOB** HeLa cells were treated with various concentrations of ABOB which were added to culture media and its viability was examined by eosin exclusion test for 7 days after the treatment. The percentages of living cells treated with 1.0 mg/ml or less concentrations of ABOB were 80 to 90 per cent as that of untreated HeLa cells during the period observed (Fig. 1). Treatment of 2.5 mg/ml ABOB, however, brought a remarkable cell damage after 7 days. Accordingly, 0.5 and 1.0 mg/ml of ABOB

Fig. 1 *Viability of Hela cells treated with ABOB.*

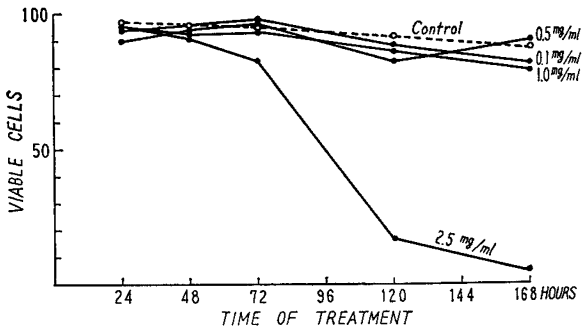
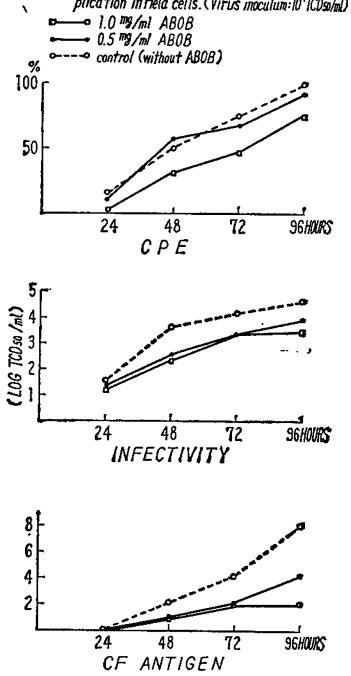



Fig. 2 *Effect of ABOB on adenovirus type 3 multiplication in Hela cells. (Virus inoculum: $10^4$ TCD50/ml)*




were used throughout the experiment.

**Contact Effect of ABOB on Adenovirus**

Adenovirus type 8 was used for the test. ABOB was added to infected culture fluid with Kanehisa strain ($10^{8.5}TCD_{50}$/ml) to give the final concentration of 2.0 mg/ml. Infectivity titration of the mixture was done after it was kept at room temperature for 24 hours. No significant changes of infectivity titer between ABOB-treated virus and control virus were demonstrated.

**Effect of ABOB on Adenovirus in HeLa Cells** As shown in Fig. 2 when HeLa cells were inoculated with $10^4$ $TCD_{50}$/ml of type 3 adenovirus, time of onset and development of cytopathic effect (CPE) at a concentration of 0.5 mg/ml of ABOB were nearly similar to the virus control. At a concentration of 1.0 mg/ml of ABOB, hewever, apparently lesser CPE was observed than the control.

Infectivity and CF antigen titers were lower than the control under the presence of ABOB 0.5 or 1.0 mg/ml.

After inoculation with $10^{8.5}$ $TCD_{50}$/ml of type 8 adenovirus, CPE (in the early stage), infectivity titer, HA titer and CF antigen titer were lower than those of the control.

The degree of decrease is more marked in 1.0 mg/ml of ABOB than in 0.5mg/ml (Fig. 3). When inoculated with smaller dosis ($10^{2.6}TCD_{50}$/ml) of type 8 virus (Fig. 4), production of HA and CF antigen was apparently inhibited in ABOB-treated HeLa cells. As regards to CPE, 150 hours after virus inoculation more remarkable CPE was observed at 1.0 mg/ml of ABOB than in the control perhaps because of cell degeneration due to ABOB itself, not to virus infection.

The correlation of initiation of ABOB treatment to inhibition of virus multiplication was studied. Treatment of ABOB was started 24 hours before, at the time of inoculation and 24 hours after virus inoculation. In HeLa cell infected with type 8 virus ($10^{8.5}TCD_{50}$/ml) HA and CF titer was lower in the presence of ABOB of 0.5 mg/ml in any onset of treatment than the control. CPE appeared at lower level only in the case of ABOB treatment 24 hours before virus inoculation (Fig. 5).

At the concentration of 1.0 mg/ml of ABOB, less CPE was observed irrespective of the time of onset of ABOB treatment than in the control. HA and CF antigen productions were apparently inhibited than in the control. The degree of inhibition is more marked in the case of 1.0 mg/ml when compared with that of 0.5 mg/ml (Fig. 6).

In general, the earlier beginning of ABOB treatment tended to bring the stronger inhibition of

Fig. 3 *Effect of ABOB on adenovirus type 8 multiplication in Hela cells. (Virus inoculum: $10^{3.5}$ TCD50/ml)*

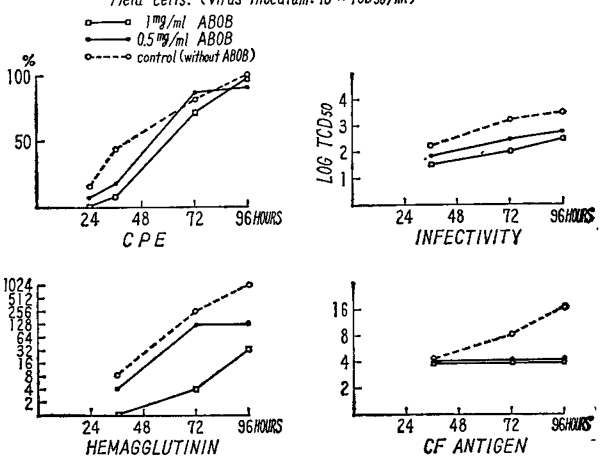


Fig. 4 *Effect of ABOB on adenovirus type 8 multiplication in Hela cells. (Virus inoculum: $10^{2.5}$ TCD50/ml)*

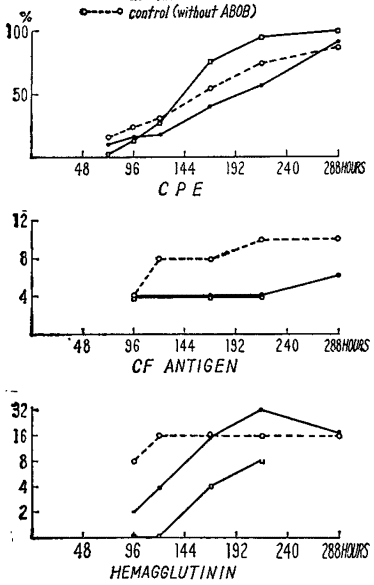



Fig. 5 *Correlation of onset of ABOB treatment and inhibitory effect on adenovirus type 8 (ABOB: 0.5 mg/ml)*

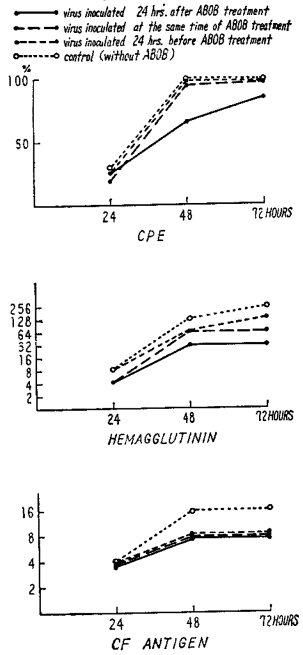



Fig. 6 *Correlation of onset of ABOB treatment and inhibitory effect on adenovirus type 8 (ABOB: 1.0 mg/ml)*

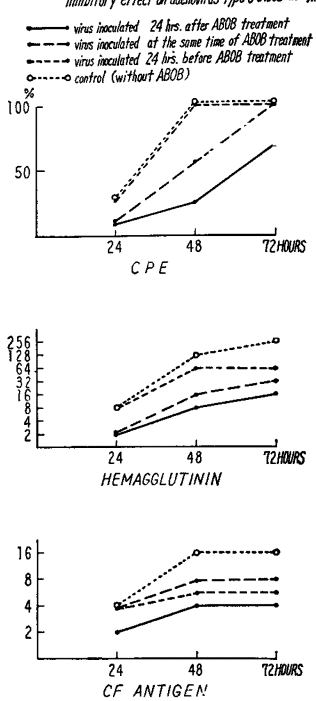



Fig. 7 *Viability of Hela cells treated with sulfisomezole.*

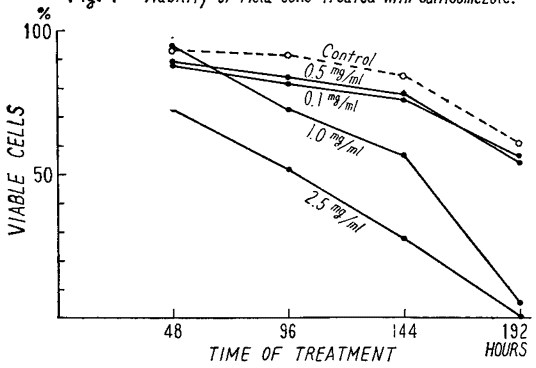
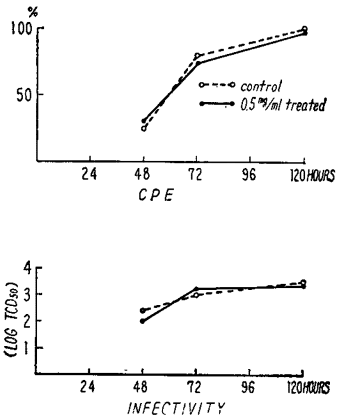



Fig. 8 *Effect of sulfisomezole on adenovirus type 8 multiplication in Hela cells. (Cells were treated with sulfisomezole 24 hours before virus inoculation. inoculum: $10^{3.5}$ TCD50/ml)*




virus multiplication in both of 0.5 and 1.0 mg/ml concentration.

A control study with sulfisomezol was made using type 8 adenovirus. Viability of HeLa cells in different concentrations of sulfisomezol was tested (Fig. 7). No inhibitory effects on CPE and infectivity titer were observed (Fig. 8).

## Discussion and Summary

The study was carried out to know whether ABOB can inhibit the adenovirus multiplication or not. HeLa cell could be used under the concentration of 1.0 mg/ml or less of ABOB for this experiments.

The results obtained suggest that adenovirus multiplication in HeLa cell was inhibited by the treatment of ABOB with the concentration of 1.0 or 0.5 mg/ml to the culture media, although no direct virucidal effects were demonstrated ***in vitro***.

The beginning of treatment of HeLa cell with ABOB was earlier, inhibitory effect of multiplication was stronger.

The mechanism of antiviral activity of ABOB remains to be studied.

## References

1) S. KAMIYA, M. KAJI & S. URATA: Isolation of adenovirus from the patient with epidemic keratoconjunctivitis. Jap. J. Clin. Exp. Med. 36, 1664, 1959.
2) S. KAMIYA: Studies on the adenovirus. Fukuoka Acta Medica, 52, 63, 1961.
3) REED, J. L. & MUENCH, H.: A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493, 1938.