Inhibition of Sendai Virus by N^1-Isobutylbiguanide Hydrochloride as Studied by Immunofluorescent Staining. (30468)

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The inhibitory effect of N^1-isobutylbiguanide hydrochloride on the growth of myxoviruses both *in vitro*(1,2) and *in vivo*(3) has been reported from this laboratory. Inhibition by the compound of Sendai virus growth in HeLa cells was demonstrated at the concentration of 62 μg/ml(1,2). Acute toxicity of the compound in mice was relatively low, and the compound showed an inhibition of the accumulation of hemagglutinin (HA) in the lung of the mice infected with influenza A (PR8) virus and also a prolongation of life span of the infected animals(2).

The present study was aimed at revealing the mechanism of action of the compound by using an immunofluorescent technique(4). Accumulated knowledge of Sendai virus growth(5,6,7) seemed to indicate that L cells, rather than HeLa cells, were better for these experiments.

Materials and methods. Virus: The Fushimi strain of Sendai virus, propagated in the allantoic cavity of 10-day eggs, was used throughout. For preparation of stock virus, 0.2 ml of infected allantoic fluid diluted to 10^{-5} was inoculated into the allantoic cavity of 10-day chick embryos and the allantoic fluids were harvested after 48 hours incubation at 35°C. The 50% egg infectious dose (EID_{50}) of the stock was $10^{10.0}$ per ml, and HA titer, $10^{3.7}$ per ml.

L cell cultures: L cells were grown in the growing medium of YLE solution plus 10% bovine serum. The YLE solution consisted of 0.5% lactalbumin hydrolysate (Difco) and 0.1% yeast extract (Difco) in Earle's solution with 0.45% glucose. For virus studies, a maintenance medium (MS) consisting of YLE solution plus 2% inactivated horse serum was used. This solution exhibited no inhibitory action on Sendai virus growth.

Experiments in tube cultures: The tubes received 3×10^5 cells in 1 ml of growing medium and 3-day-old cultures were used. The compound, N^1-isobutylbiguanide hydrochloride, was dissolved in MS to give a concentration of 62 μg/ml. To investigate the inhibitory effect of the compound on Sendai virus growth in L cells, the tubes were divided into 4 groups and treated in the following manner: A. Control tubes were washed and inoculated with 1 ml of virus in MS at a multiplicity of about 100 EID_{50} per cell and this time was considered 0 hour. After an adsorption at 37°C for 60 minutes, the inoculum was removed and the tubes were washed with Hanks' solution, then refed with fresh MS. B. Tubes were treated with the compound between —24 hours and the time of infection, with subsequent steps the same as controls. C. The compound was present between —24 and 24 hours. At the end of the virus adsorption period, the tubes were washed and refed with fresh MS containing the compound after 24 hours, and the drug-containing medium was replaced by normal MS. D. The tubes received the compound and virus at 0 hour simultaneously. After the adsorption, the inoculum was removed and the tubes were refed with fresh MS containing the compound, which was present throughout the experiment.

The amount of HA released in the culture fluid was assayed by the standard pattern method. Cytopathic effect (CPE) of the virus and the hemadsorption of the infected cells were also examined to estimate the degree of inhibition of virus multiplication.

Experiments in coverslip cultures: Coverslip cultures were used to follow the process of viral multiplication by immunofluorescent technique. Details of the technique are being reported elsewhere(8). Three-day L cell coverslip cultures grown in a Petri dish were treated with the compound following an experimental schedule similar to the culture experiment. To infect the coverslips, a 0.05 ml of 10^{-4} dilution of stock virus was layered on each coverslip and the incubation

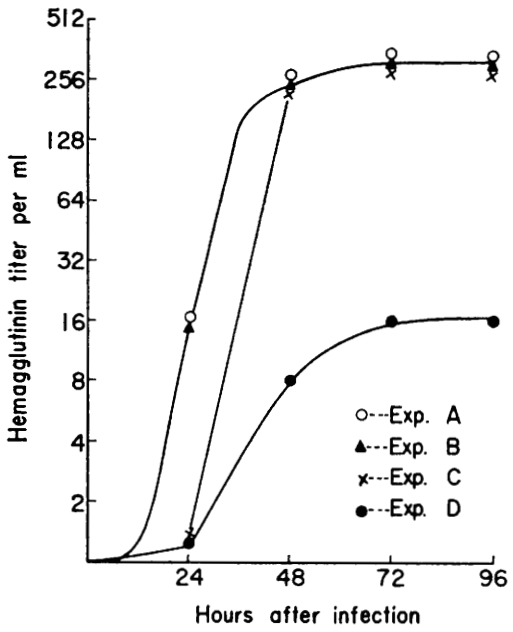

FIG. 1. Effect of N¹-isobutylbiguanide hydrochloride on production of Sendai virus in L cell tube cultures. Group A: Untreated culture. Group B: Treated (62 μg/ml) between —24 and 0 hr. Group C: Treated between —24 and 24 hr. Group D: Treated between 0 and 96 hr. Details in text.

was carried out at 37°C in an atmosphere of 5% CO_2 in the air. Specimens were taken out every 24 hours and stained with fluorescent antibody as described(4). Virus titers in the culture fluids were also measured by egg infectivity, using 4 eggs per dilution.

Results. Inhibition in tube cultures: The experiment was performed according to the schedule described above. The results are illustrated in Fig. 1. Each group of the experiment consisted of 20 tubes of L cell cultures. Five tubes were taken out from each group every 24 hours, assayed for HA titer in the culture fluid and examined for CPE and hemadsorption.

The HA titer in the culture fluid was reduced to 1/16 of the control when the compound was present from 0 to 96 hours post infection (D). No effect was observed, however, when treatment was done only for 24 hours prior to virus inoculation (B). Treatment from —24 to 24 hours resulted in reduction of the HA titer at 24 hours but no reduction at all at 48 hours after (C).

The characteristic CPE of Sendai virus on L cells is rounding(5). At 24 hours almost all of the control cells are rounded with positive hemadsorption, whereas only a limited number of treated cells showed rounding tendency, and they were almost negative in hemadsorption test at 24 hours. At 96 hours after infection, control cells were sloughing off, whereas treated cells remained on the glass wall but with increased granulation in cytoplasm.

Immunofluorescent studies in coverslip cultures: The purpose of this experiment was 2-fold: to determine (1) whether the number of infectious centers was reduced in presence of the compound; and (2) what stage of viral antigen development was inhibited by the compound.

The results are summarized in Table I. Four developmental stages were differentiated in the control culture(7,8). In the first stage, fine fluorescent granules were visible only in the juxtanuclear region. In the second, cytoplasm surrounding what one might guess to be the Golgi area was the main site of antigen accumulation, but the structure of both cytoplasm and nucleus was well-preserved (Fig. 2). In the third stage, the anti-

TABLE I. Effect of N¹-Isobutylbiguanide on Development of Sendai Virus Antigen in L Cells as Revealed by Immunofluorescent Staining.

		No. of fluorescent cells													
		24 hr					48 hr					72 hr				
Group	Duration of treatment (hr)	Stage* I	II	III	IV	Total	Stage I	II	III	IV	Total	Stage I	II	III	IV	Total
A	Untreated	0	20	13	0	33	0	0	25	10	35	0	0	0	35	35
B	−24 to 0	0	21	9	0	30	0	4	22	7	33	0	0	4	30	34
C	−24 to 24	14	3	0	0	17	3	25	6	0	34	0	3	11	18	32
D	0 to 96	13	3	0	0	16	7	20	0	0	27	7	26	0	0	33

* See text.

gen granules gradually moved towards the cell margin, the cytoplasm being full of antigen masses with intense immunofluorescence (Fig. 3). In the fourth stage, the fluorescent antigens were distributed around the cell surface, suggesting the feature of virus release (Fig. 4). Cell membrane was almost destroyed at this stage.

In the cultures treated with the compound from 0 to 96 hours (Group D), a significant

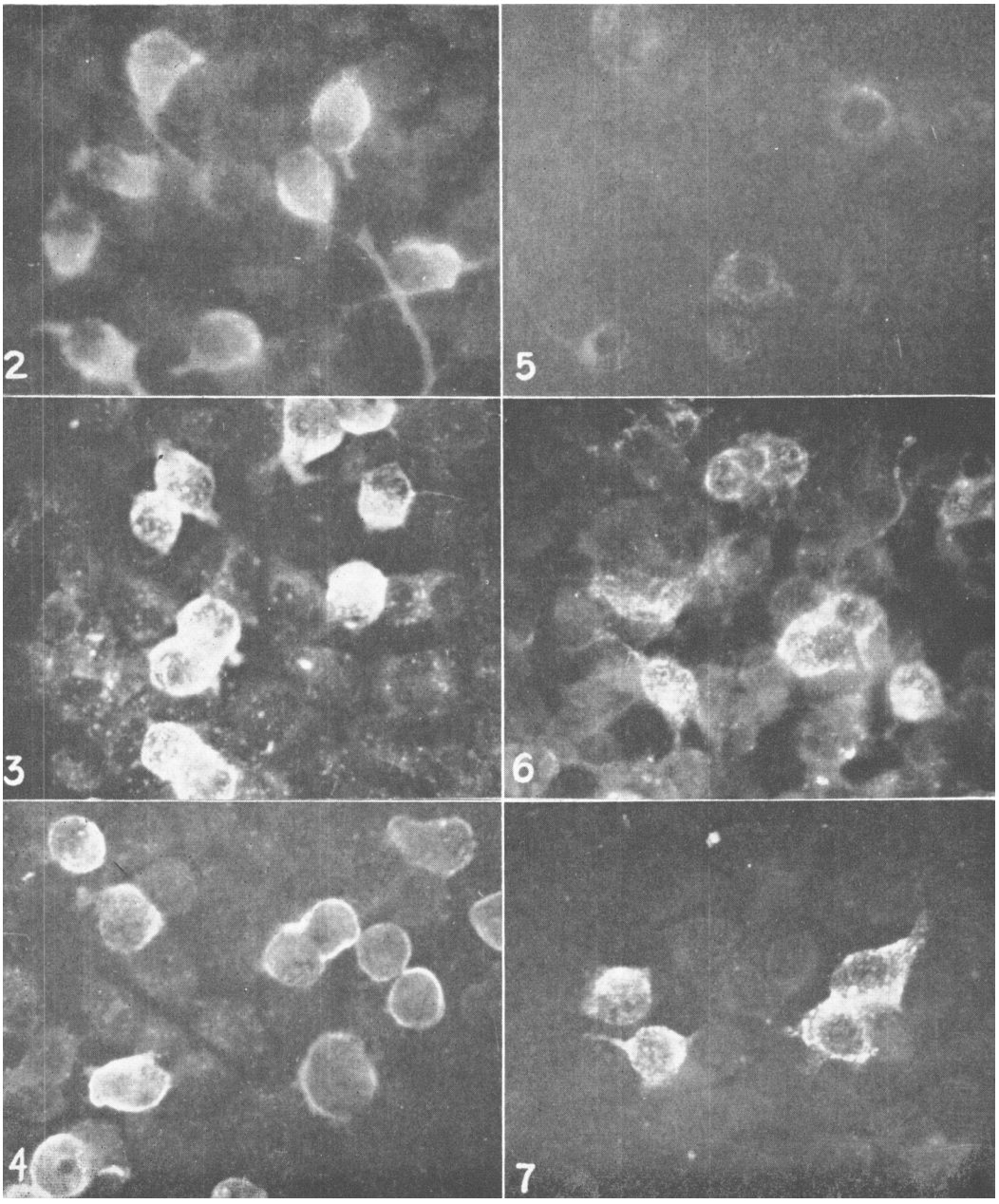

FIG. 2 to 7. Immunofluorescent antigen development in L cells infected with Sendai virus in absence or presence of N^1-isobutylbiguanide hydrochloride. Fig. 2, 3, 4. Untreated cultures; 24, 48, and 72 hr respectively. Fig. 5, 6, 7. Compound added at 0 hr and present throughout infection; 24, 48, and 72 hr respectively.

delay in the process of viral antigen synthesis was observed at 24 hours. The viral antigens in the control exhibited typical stage II configuration, whereas in the treated cultures only fine, granular fluorescence, locating juxtanuclearly, was observed (stage I), this feature being comparable to that of the control at 12 hours (Fig. 5). The total number of fluorescent cells was approximately one-half that in the control at 24 hours (Table I). At 48 hours, the cytoplasmic fluorescence in the control gradually moved towards the cell margin (stage III), whereas the fluorescent cells in the treated culture were still at the first or the second stage (Fig. 6), although the total fluorescent cell number somewhat increased when compared to that at 24 hours. At 72 hours, the number of fluorescent cells in the treated group was not significantly different from that of the control, but all of the fluorescent cells were still at the stage II or I (Fig. 7). Virus titers in the control culture fluids were $10^{3.4}$, $10^{5.2}$, and $10^{5.7}$ EID_{50} per ml at 24, 48, and 72 hours respectively, whereas those of the treated were $10^{2.4}$, $10^{4.4}$, and $10^{4.2}$, EID_{50} per ml. This reduction rate was compatible with HA reduction rate obtained in the tube culture experiment.

When the compound was removed 24 hours after virus inoculation (Group C), antigen development at 48 hours was less advanced than in control at 24 hours. However, at 72 hours, *i.e.*, 48 hours after removal of the compound, antigen development was more advanced than that of the control at 48 hours (Table I).

When the cells were pretreated with the compound for 24 hours only (Group B), no significant differences from the control were found (Table I).

Discussion. The inhibitory effect of N^1-isobutylbiguanide hydrochloride on Sendai virus growth in L cells was clearly demonstrated in the present study by examining HA titers released in the tube culture and EID_{50} titers in the fluid of coverslip cultures. The effective concentration of the compound in L cells was the same as in HeLa cells(2), and the degree of reduction in HA was also the same between these 2 different cell cultures.

Immunofluorescent staining of viral antigens inside the cell can be a useful tool in revealing the site of action of antiviral agents. This technique was used in the present study primarily to learn whether the number of infectious centers was reduced in the presence of the compound(9). The results indicate that the number of infectious centers was not reduced by the compound even when the compound was maintained through the whole course of infection. Since virus grown in L cells is not infectious to L cells(5,6), the infectious centers counted by immunofluorescent staining should be those produced by the inoculum virus. Thus it is evident that in L cells the compound did not work to reduce the number of infectious centers through inhibition of adsorption or penetration of the virus. The compound is already known to be not virucidal even at high concentrations such as 1 mg/ml(1).

The initial synthesis of viral antigens in the treated cells followed a pattern similar to controls, though somewhat delayed. The syntheses of both soluble and viral antigens in L cells are known to occur within 12 hours after infection(5). Immunofluorescent features of the treated cells at 24 hours were comparable to 12-hour features of the controls. Immunofluorescent features at 72 hours in the treated cells remained almost the same as at 48 hours, *i.e.*, similar to 24 hours in the control. Thus as far as the synthesis of early antigens is concerned, treated cells followed almost the same pattern as control cells, whereas the amount of virus in the treated cultures at 24 hours was only 1/16 of the control in HA and 1/10 in infectivity. The same reduction rate was obtained at 48 hours and 72 hours respectively. When these results are taken together, the effect of N^1-isobutylbiguanide hydrochloride can be explained as the inhibition of the final synthesis of virus particles in each cell to 1/10 of the control.

Another interesting characteristic of the effect of the compound was that the action was reversible. Once the compound was removed, an immediate virus synthesis was shown by means of HA titration and immunofluorescent staining. Although the compound seemed to inhibit the late maturation

process as stated above, the mechanism of the action might be quite different from that of proflavine in a sense that the action is reversible.

Summary. In the presence of a non-toxic concentration of N^1-isobutylbiguanide hydrochloride, HA production in L cells infected with Sendai virus was reduced to 1/16 of the control. The effect of the compound was reversible, and the presence of the compound through the entire course of infection was necessary and essential for the inhibition. The number of infectious centers was not reduced even when the cell was treated with the compound all through infection, indicating that the compound did not affect the processes of adsorption or penetration of the virus. However, the infected cells remained in the first stage of antigen development in the presence of the compound, *i.e.*, the accumulation of antigens was limited to the juxtanuclear region of cytoplasm and not extended to the entire cytoplasm. It is concluded that the effect of the compound is on the inhibition of the late maturation mechanism of Sendai virus synthesis.

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An Ovarian Ascorbic Acid Depletion Factor in Starch Gel Preparations Following Electrophoresis.* (30469)

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In studying ovarian ascorbic acid depletion (OAAD) activity in the plasma of hypophysectomized cockerels(1), an attempt was made to use starch gel electrophoresis to separate and purify plasma LH. This approach led to an investigation of the OAAD activity in the starch gel preparation itself.

Materials and methods. Starch gels were prepared as reported by Ferguson and Wallace(2) with certain modifications. Partially hydrolyzed potato starch (Connaught Medical Research Laboratories, Toronto) was suspended in a buffer composed of 90% 0.0033 M citric acid, 0.016 M Tris, and 10% 0.02 M lithium hydroxide, 0.076 M boric acid, with a pH at 20°C of 8.1. After heating properly, the gel was poured into a mold on a glass plate formed by plexiglass strips, which were held in place by clips for a final mold size of 12 × 21.7 × 0.6 cm. A mold with 8 slot-formers equidistantly spaced, was placed on the hot gel in such a way as to form 8 slots, 1.2 cm wide, 0.5 cm deep, and 0.1 cm thick, 5 cm from one end of the gel. The gel was cooled at 5°C for at least 30 minutes, the mold carefully removed, hot white petroleum jelly added to the area about each slot, and then 3 drops of the sample material injected into each slot. The entire surface of the gel was then covered with hot petroleum jelly, after which a 2-cm strip of the cooled jelly was removed from each end to allow contact with the tray buffer wicks. Departing from Ferguson and Wallace(2), the tray buffer was 0.0286 M lithium hydroxide and 0.19 M boric acid, which was placed in the cathodal trays and in the anodal tray directly connected to the

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