Inhibition of Enterovirus Cytopathic Effects by 2-(α -Hydroxybenzyl)-Benzimidazole¹

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ABSTRACT

BABLANIAN, ROSTOM (The Rockefeller University, New York, N.Y.), HANS J. EGGERS, AND IGOR TAMM. Inhibition of enterovirus cytopathic effects by 2-(α -hydroxybenzyl)-benzimidazole. *J. Bacteriol.* **91**:1289-1294. 1966.—2-(α -Hydroxybenzyl)-benzimidazole (HBB), a specific inhibitor of enterovirus multiplication, markedly delayed the development of cytopathological changes induced by echovirus 12 or coxsackievirus B4 in monkey kidney cells, but did not prevent the ultimate degeneration of infected cells, even though virus multiplication was inhibited. The study of the development of viral cytopathic effects was facilitated by the use of antiviral immune serum, which restricted the infection to those cells which became infected by the inoculated virus and thereby established single-cycle conditions. With echovirus 12 and coxsackievirus B4 not all cells could be infected initially, even when cultures were inoculated at input multiplicities in excess of 100 plaque-forming units per cell.

2-(α -Hydroxybenzyl)-benzimidazole (HBB) is a specific inhibitor of the reproduction of many enteroviruses (8, 13, 14). Echoviruses and coxsackieviruses are more sensitive to HBB than are polioviruses (8, 15). HBB inhibits the synthesis of viral ribonucleic acid (RNA) polymerase (3). The available evidence indicates that inhibition of viral RNA and capsid protein synthesis is secondary to inhibition of the formation of the enzyme required for the synthesis of viral nucleic acid (3, 10). Possible effects of HBB on the development of virus-induced cytopathic changes in infected cells under single-cycle conditions have not been reported. Inhibition of viral cytopathic effects by HBB in multiple-cycle experiments (8) may be largely due to prevention of spread of infection through inhibition of virus production in the few cells infected by the inoculated virus (16).

In the present study, we have found that HBB, like guanidine in the case of poliovirus (1, 2), delays the appearance of virus-induced morpho-

logical changes in the infected cells, but does not prevent their ultimate degeneration. The delayed degeneration occurs in the absence of virus multiplication.

MATERIALS AND METHODS

Viruses. Echovirus type 12 (Travis) and coxsackievirus B type 4 [Powers, antibody-sensitive variant (4)] seeds were prepared in primary cultures of monkey kidney cells.

Cell cultures and media. Primary cultures of monkey kidney cells were prepared in the following way. Rhesus monkey kidneys were trypsinized, and plastic petri dishes (60 by 15 mm) were seeded with 5 ml of a 1:300 suspension of cells in growth medium. The growth medium consisted of Hanks balanced salt solution (11) with 0.5% lactalbumin hydrolysate and 2% calf serum, which had been inactivated at 56 C for 30 min. Four days after seeding the cells, the medium was removed from the plates, and fresh growth medium was introduced. Complete monolayers of cells were obtained in the petri dishes on the 5th or 6th day after plating. For maintenance of cells, Eagle's (6) medium was used. Phosphate-buffered saline (PBS) (5) was used to wash off virus from infected plates. All media contained penicillin (500 units per ml), streptomycin (0.1 mg/ml), and nystatin (25 units per ml).

Immune sera. Immune serum against echovirus 12 and coxsackievirus B4 was prepared by injecting rabbits intravenously with 10 ml of stock virus two times, with an interval of 7 days between the injections. Rabbits were bled 24 days after the first in-

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jection, and both normal rabbit serum, obtained before immunization, and antiviral immune serum were inactivated at 56 C for 30 min.

Compound. HBB was obtained through the courtesy of Arthur F. Wagner of Merck Sharp and Dohme Research Laboratories, Rahway, N.J.

Measurement of virus. Virus was measured by plaque assay employing primary monkey kidney cell monolayers (9). To measure total virus, the medium and cells from infected cultures were collected, frozen and thawed three times, centrifuged to remove cellular debris, and assayed. To measure released virus, the medium from infected cultures was collected, centrifuged, and the supernatant fluids were used for assay.

Quantitation of virus-induced cell damage. The morphological lesions that result from echovirus 12 and coxsackievirus B4 infection were observed in an inverted phase-contrast microscope. Vacuolated, retracted, and rounded cells were considered damaged. An average of 2,000 cells, which included both damaged and morphologically normal cells, were counted per experimental group. The extent of virus-induced cell damage was expressed as per cent of cells affected.

RESULTS

Infection of monkey kidney cells by echovirus 12 and coxsackievirus B4. To determine the proportion of cells infected by the inoculated echovirus 12 or coxsackievirus B4, we limited the infection to a single cycle by the use of specific immune serum. Monkey kidney cells grown in plastic petri dishes (60 by 15 mm) were inoculated with 0.5 ml of echovirus 12 or coxsackievirus B4 in Eagle's medium at a multiplicity of 18 plaque-forming units (PFU) per cell. After 1-hr adsorption, the inoculation medium was removed, and the plates were washed with PBS. Each culture then received 2.5 ml of Eagle's medium. After 1 hr Eagle's medium was removed from groups of cultures, and specific immune serum against echovirus 12 (diluted 1:20 in Eagle's medium) or coxsackievirus B4 (1:30 in Eagle's medium) was added. At 48 hr after infection the cultures were examined for virus-induced damage, and the medium was collected for virus assay. Plaque assays indicated that the immune sera were capable of neutralizing completely all unadsorbed and released virus.

Experiments of this type showed that, at an input multiplicity of 18 PFU/cell, only 21 to 31% of cells in echovirus 12-infected cultures became infected, that is, underwent virus-induced degenerative changes; in coxsackievirus B4-infected cultures, the proportion was even smaller, varying between 11 and 14% in different experiments.

Increasing the multiplicity severalfold resulted only in a moderate increase in the proportion of cells which became infected by the inoculated echovirus 12. This is illustrated in Table 1, which

shows that, even at a multiplicity of 250, only 50% of the cells became infected. The reliability of the above method in determining the proportion of infected cells was confirmed with echovirus 12 by employing the "infectious center" technique. With the infectious-center technique (7), echovirus 12 at a multiplicity of 39 PFU/cell infected 33% of the cells in a monolayer.

Effect of HBB on echovirus 12-induced cytopathological changes. Table 2 shows the results of an experiment in which the rate of development of echovirus 12-induced cellular changes was followed both in the presence and absence of HBB in cultures of monkey kidney cells inoculated at a virus-cell multiplicity of 62. Immune serum was added 2 hr after infection to limit infection to a single cycle (column 2, Table 2). HBB, added 1 hr after infection, greatly delayed the virus-induced morphological changes (column 3, Table 2). At 9 hr after infection, no significant virus-induced cytopathological changes were observed in HBB-treated cultures, whereas 47% of cells had become rounded in the absence of HBB. By 19 hr after infection, only a small percentage of the cells showed virus-induced damage in

TABLE 1. Effect of multiplicity on infection of monkey kidney cells by echovirus 12

Treatment of cultures	Per cent damaged cells in infected cultures (48 hr after infection)			
	Multiplicity (PFU/cell)			
	250	50	25	18
Echovirus 12 immune serum added 2 hr after infection	50	31	37	23
None	100	100	100	100

TABLE 2. Delay of echovirus 12-induced cell damage in the presence of HBB*

Time after infection	Per cent damaged cells in infected cultures			
	Echovirus 12; immune serum added 2 hr after infection	Echovirus 12; HBB added 1 hr after, and immune serum 2 hr after, infection	Echovirus 12	Echovirus 12; HBB added 1 hr after infection
hr				
9	47	3	44	2
19	41	9	68	7
39	34	29	93	29

* Primary cultures of monkey kidney cells were infected with 0.5 ml of echovirus 12 at a multiplicity of 62 PFU/cell. The final dilution of echovirus 12 immune serum was 1:20. The concentration of HBB was 220 μ M.

HBB-treated cultures, but by 39 hr after infection the majority of the initially infected cells had become rounded. As expected, the effect of HBB on the development of cytopathological changes in the absence of immune serum was the same as in its presence. In infected cultures which received neither HBB nor immune serum, there was evidence of spread of infection at both 19 and 39 hr. Plaque titrations indicated that no infectious virus was produced in HBB-treated cultures. We concluded that, although HBB greatly delays virus-induced morphological changes in infected cells, it does not prevent their ultimate occurrence.

In other experiments, the development of cell rounding in echovirus 12-infected HBB-treated cultures was followed for a period of 7 days in the absence of immune serum. The cultures were inoculated at a multiplicity of 18, which results in infection of 21 to 31% of cells (see above). Figure 1 shows the mean results of three such experiments. The untreated cultures were followed only for 2 days, because 87% of the cells already showed virus-induced damage at 24 hr after virus inoculation, and nearly all cells showed cytopathic effects in 2 days. Clearly, the

FIG. 1. Effect of 2-(α -hydroxybenzyl)-benzimidazole (HBB) on the development of echovirus 12-induced morphological damage in monkey kidney cells. Primary cultures of monkey kidney cells were infected with 0.5 ml of echovirus 12 at a virus-cell multiplicity of 18 PFU/ml. The cultures consisted of complete monolayers of cells, approximately 2×10^6 cells per 60-mm petri dish. After 1 hr of adsorption, the virus inoculum was removed and cultures were washed with warm PBS. Each plate then received 2.5 ml of Eagle's medium with or without 220 μ M HBB, and the cultures were further incubated at 36 C. In a defined area of the dish, the rounded and the flat cells were counted, and the proportion of rounded cells was expressed as per cent affected cells. Uninfected cultures served as controls for rounding of cells due to causes other than infection. The values for virus-damaged cells in infected cultures were obtained by subtraction of the number of rounded cells seen in uninfected cultures from the number of rounded cells in the infected cultures.

virus produced in the initial cycle infected other cells, and, in the absence of HBB, the cycle was repeated until all cells had become infected and degenerated. In contrast, in HBB-treated echovirus 12-infected cultures the proportion of damaged cells was only 17% at 24 hr, and, at 48 and 72 hr, it was 33 and 37%, respectively, clearly indicating that up to and including the third day morphological changes developed only in the proportion of cells initially infected by the inoculated virus. However, on the 6th and 7th days after infection, 65 and 72% of the cells were affected, respectively. Because of this apparent late spread of infection, it was of interest to determine whether multiplication of virus had occurred in spite of the presence of HBB. Comparative studies of virus production and development of virus-induced cell damage were carried out. Figure 2 shows the yields of echovirus 12 on consecutive days in the presence of HBB, and the percentage of affected cells in such treated, infected cultures. As in the previous experiment, the percentage of damaged cells in HBB-treated cultures reached about 30 within a period of 3 days after infection. By the 7th day after infection, 82% of the cells were damaged, a value similar to that observed in the previous experiment. There was no multiplication of the virus detected in the HBB-treated cultures in the first 3 days. However, by the 4th day after infection, and thereafter, an increase in infectious echovirus 12 was detected in spite of the presence of HBB from the beginning of the experiment.

On the basis of previous studies (15), it seemed probable that the echovirus 12 which succeeded in growing in the presence of HBB was probably a resistant mutant. To establish this, the 7th day yields of echovirus 12 from untreated cultures were titrated by the plaque procedure on monolayers of monkey kidney cells with or without HBB in the agar overlay.

Echovirus 12 from untreated control cultures failed to form plaques when the overlay contained HBB (Table 3). In contrast, the virus which had grown in HBB-treated cultures was able to form plaques both in the presence and absence of HBB in the overlay, and was therefore drug-resistant.

Effect of HBB on coxsackievirus B4-induced cytopathological changes. In the following experiment, the development of cytopathological changes in coxsackievirus B4-infected HBB-treated cultures was followed for a period of 7 days. The cultures were inoculated at a multiplicity of 18 PFU/cell, which results in the infection, on the average, of 13% of cells (see above). Figure 3 shows that in coxsackievirus B4-infected untreated culture 49% of the cells became damaged within 24 hr, and, by 48 hr after

FIG. 2. Effect of a 2-(α -hydroxybenzyl)-benzimidazole (HBB) on echovirus 12 multiplication and virus-induced morphological damage in monkey kidney cells. Primary cultures of monkey kidney cells were infected with echovirus 12 at a multiplicity of 18 PFU/cell and treated with HBB as described in Fig. 1. After recording virus-induced cell damage daily, the cultures were stored at -20°C . At the end of the experiment, total virus from the cultures was measured by the plaque technique. Values for virus-damaged cells were obtained by subtraction of the number of rounded cells seen in uninfected cultures from the number seen in the infected cultures.

TABLE 3. Emergence of HBB-resistant echovirus 12 after prolonged incubation of infected cultures with HBB

Virus*	Plaque titration (PFU/ml)	
	Regular overlay†	Overlay with 100 μM HBB
Echovirus 12 from control cultures incubated for 2 days.....	4×10^8	$<1.0 \times 10^4$
Echovirus 12 from cultures treated with 220 μM HBB and incubated for 7 days.....	1.0×10^7	1.4×10^7

* See Fig. 1.

† Difco agar in reinforced Eagle's medium with 2% calf serum.

FIG. 3. Effect of 2-(α -hydroxybenzyl)-benzimidazole (HBB) on the development of coxsackievirus B4-induced morphological damage in monkey kidney cells. Experimental conditions as in Fig. 1.

infection, over 90% of the cells were damaged. Infected and HBB-treated cultures, however, showed no virus-induced morphological changes on the 1st day of infection, and, between the 3rd

and 7th day, the degree of virus-induced cell damage remained constant at about 10%. This indicates that HBB delayed, but did not prevent, the development of virus-induced cytopathological changes.

Figure 4 shows that no multiplication of coxsackievirus B4 occurred in HBB-treated cultures over the entire period of 7 days. The cytopathic changes in this experiment also showed no significant increase after the 3rd day. It thus appears that in coxsackievirus B4 virus-infected HBB-treated cultures only the initially infected cells showed morphological changes. The extent of this damage remained constant as HBB prevented multiplication of the inoculated virus and thereby the spread of infection, and, at the multiplicity used, no drug-resistant mutants emerged capable of multiplying in the presence of the inhibitor.

Comparative study of the effects of specific immune serum and HBB on cell damage induced by echovirus 12 and coxsackievirus B4. Table 4 summarizes the results of experiments in which a direct comparison was made of the effects of HBB and of antiviral immune serum on the extent of virus-induced cell damage in cultures infected with echovirus 12 or coxsackievirus B4. It is clear that on the 3rd day after infection with either virus HBB and specific antiviral immune serum limited the involvement of an infected culture to a similar degree, although by entirely different mechanisms. These results contribute

additional evidence that HBB does not prevent the ultimate degeneration of infected cells, although, as shown above, it does delay the virus-induced morphological changes.

TABLE 4. Comparison of the effects of immune serum and HBB on the extent of morphological changes in monkey kidney cells induced by echovirus 12 or coxsackievirus B4*

Virus	Per cent damaged cells in infected cultures (72 hr after infection)		
	Eagle's medium	Immune serum added 2 hr after infection	HBB added 1 hr after infection
Echovirus 12.....	97	33	36
Coxsackievirus B4...	93	8	12

* Primary cultures of monkey kidney cells were infected with 0.5 ml of either echovirus 12 or coxsackievirus B4 at a multiplicity of 18 PFU/cell. After 1-hr adsorption, the inoculum was removed, and 2.5 ml of Eagle's medium was added for 1 hr to all cultures. The medium was then removed, and the cultures received 2.5 ml of control Eagle's medium, echovirus 12 immune serum (1:20), coxsackievirus B4 immune serum (1:30), or 220 μ M HBB in Eagle's medium.

FIG. 4. Effect of 2-(α -hydroxybenzyl)-benzimidazole (HBB) on coxsackievirus B4 multiplication and virus-induced morphological damage in monkey kidney cells. Experimental conditions as in Fig. 2.

DISCUSSION

Monkey kidney cells exposed to echovirus 12 or coxsackievirus B4 did not all become infected initially, even at high virus-cell multiplicities. However, by preventing the spread of infection in cell cultures with specific antiviral immune sera, it was possible to establish quantitatively the proportion of infected cells in cultures inoculated at varying multiplicities. This allowed the study of the effect of HBB on the development of virus-induced morphological changes in the proportion of cells initially infected by the inoculated virus, and the effect of HBB on the spread of infection in the culture. We have found that HBB delays the development of viral cytopathic effects in the cells infected with either echovirus 12 or coxsackievirus B4. The delay is of the order of 35 hr.

In echovirus 12-infected HBB-treated cells, there emerged a population of HBB-resistant particles on continued incubation of cultures. Prior to the emergence of HBB-resistant particles, only the initially infected cells underwent delayed cytopathological changes. However, with the emergence of resistant virus the infection spread rapidly, and nearly all cells in the culture became involved and degenerated. In coxsackievirus B4 infection, however, no HBB-resistant mutants appeared in the treated cultures, and only those cells which were infected by the virus in the inoculum ultimately degenerated.

Thus, HBB, like guanidine in the case of poliovirus (1, 2, 12), delays the development of virus-induced morphological changes in infected cells. The precise mechanism whereby delayed viral cytopathic effects occur in poliovirus-infected guanidine-treated cells is not clear. They may be a consequence of the early virus-induced metabolic inhibitions of cellular RNA and protein synthesis, which are not prevented by guanidine (1). An alternative possibility is the occurrence in the treated cells of slow synthesis and accumulation of viral proteins (10), resulting in delayed cytopathological changes in the absence of replication of viral genetic material. Similar arguments may be advanced to explain the delayed development of virus-induced cytopathic effects in HBB-treated infected cells with echovirus 12 or coxsackievirus B4. Further work is necessary to substantiate either hypothesis.

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