INHIBITION OF RELEASE OF VACCINIA VIRUS BY N_1-ISONICOTINOYL-N_2-3-METHYL-4-CHLORO-BENZOYLHYDRAZINE

By NOBUO KATO,* M.D., HANS J. EGGERS, M.D., and HEINRICH ROLLY, Ph.D.

(From Institut für Virologie, Justus Liebig-Universität, Giessen, and Farbwerke Hoechst AG, Hoechst, Germany)

(Received for publication 21 October 1968)

It has been found that N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH, see Fig. 1) inhibits the multiplication and the cytopathic effects of vaccinia virus in chick embryo fibroblasts and monkey kidney cells. Virus-inhibitory concentrations of IMCBH do not cause significant morphologic changes in uninfected cells. The present communication describes these findings and the effect of IMCBH on the multiplication cycle of vaccinia virus. Evidence will be presented that IMCBH inhibits the release of vaccinia virus from the infected cell.

Materials and Methods

Compound.—N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) was synthesized by Dr. E. Winkelmann, Farbwerke Hoechst AG, Hoechst, Germany. IMCBH is difficult to dissolve in cold water but dissolves to a limited extent in hot water. IMCBH suspended in Eagle's minimum essential medium (MEM) (1) at a concentration of 200 μg/ml was dissolved by heating of the suspension at 80°C for 1 hr. This stock solution was diluted further with the same medium to give the desired concentrations.

Viruses.—Three strains of vaccinia virus (P71, MPI, and IHD) were used. The P71 strain was obtained from the Virological Laboratory of Farbwerke Hoechst AG, Hoechst, Germany; the MPI strain from Dr. H. Gerth, Hygiene-Institut, Tübingen, Germany; the IHD strain (2) from Dr. S. Dales, The Public Health Research Institute of the City of New York, N.Y. The P71 and MPI strains were grown in chick embryo fibroblasts. The IHD strain, which had been passaged in L cells in Dr. Dales' laboratory, was grown in L cells.

Sindbis, vesicular stomatitis, Newcastle disease (Miyadera), fowl plague (Rostock), polio type 1 (Mahoney), ECHO type 12 (Travis), reo type 3 (Dearing), and adeno type 7 virus were also tested. Sindbis and vesicular stomatitis virus were propagated in chick embryo fibroblasts, Newcastle disease and fowl plague virus in embryonated eggs, and the other viruses in monkey kidney cells.

Cell Cultures.—Chick embryo fibroblasts were prepared from 11 day-old embryos by trypsinization according to Kraus and Schäfer (3). They were grown in plastic Petri dishes (60 × 15 mm, Greiner, Nürtingen, Germany) or in screw cap glass tubes. Dishes and loosely

* Recipient of a Dozentenstipendium from the Alexander von Humboldt-Stiftung. Permanent address: Department of Bacteriology, Nagoya University School of Medicine, Nagoya, Japan.

closed tubes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. When used after overnight incubation, Petri dishes contained approximately 4×10^6 cells per dish.

Tube cultures of rhesus monkey kidney, L, and BHK21 cells were also used. Rhesus monkey kidney cells were obtained by trypsinization. The growth medium for monkey kidney cells consisted of Eagle's MEM with 5% heat-inactivated fetal bovine serum, the growth medium for L and BHK21 cells of reinforced Eagle's medium (4) with 10% heat-inactivated fetal bovine serum.

Eagle's MEM without serum was used as maintenance medium for all cell cultures.

Virus Titrations in Tube Cultures.—Serial 10-fold dilutions of virus were made and 0.1 ml aliquots of each dilution were inoculated into groups of three tubes containing 1.9 ml of maintenance medium. The cultures were incubated at 37°C in a stationary position. They were observed at least every 2nd day for the development of cytopathic effects; the final readings were made 5 days after virus inoculation. The amount of infective virus was expressed in terms of 50% tissue culture infective doses ($TCID_{50}$).

Plaque Assay.—In most experiments, measurement of infective virus was carried out by plaque assay. Plastic Petri dishes with confluent monolayer cultures of chick embryo fibroblasts were incubated with 0.2 ml of serial 10-fold dilutions of virus in Lavit medium (3).

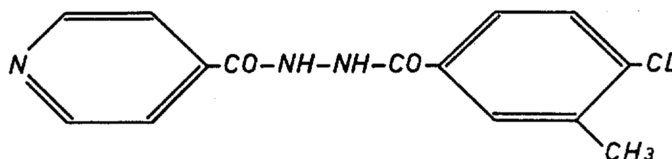

FIG. 1. Structure of N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine.

Two dishes per dilution were used. After an adsorption period of 1 hr at 37°C, the cell sheet was overlaid with 4 ml of agar overlay. The overlay consisted of equal volumes of 1.4% agar and two times concentrated Lavit medium. Cultures were incubated for 4 days at 37°C in 5% CO_2. Then, 0.5 ml of 0.05% neutral red in water was added to each culture. Plaques were counted 3 hr later. 1 plaque-forming unit (PFU) of vaccinia virus (P71) was found to correspond to approximately 3 $TCID_{50}$.

Growth Curve Experiments.—Replicate chick embryo cell monolayers in plastic Petri dishes were washed with phosphate-buffered saline (PBS) (5) and inoculated with 0.5 ml of virus contained in medium with or without IMCBH. After adsorption for 2 hr at 37°C in 5% CO_2, the inoculum was removed, the monolayers were washed twice with warm PBS with or without IMCBH, 4 ml of Eagle's MEM with or without IMCBH was added, and the dishes were incubated at 37°C in 5% CO_2. At intervals, four dishes were removed from the incubator. For determination of released virus, the fluid medium of two dishes was collected, centrifuged at 3000 rpm for 10 min, and the supernatant stored at −35°C until assayed. The two other dishes were placed at −35°C. Subsequently, the cultures were thawed, the cell sheets were scraped from the plastic surface, and the cells with their supernatant medium were harvested. Samples were rapidly frozen and thawed three times, and centrifuged at 3000 rpm for 10 min to remove debris. The supernatant was stored at −35°C until assayed. These samples were used for assay of the total amount of virus produced. For determination of cell-associated virus, two dishes of cultures from which fluid medium had been removed were washed twice with PBS, 4 ml of Eagle's MEM was added to each dish, and the dishes were placed at −35°C. Subsequently, the cultures were treated in the same way as the samples used for assay of total

virus. Measurement of infective virus was performed by plaque assay. The time of virus inoculation was considered zero time.

RESULTS

Toxicity of IMCBH to Chick Embryo Fibroblasts.—IMCBH did not cause any toxic morphologic changes in all kinds of cells used in the present study up to a concentration of 50 μg/ml. The minimal concentration to cause detectable morphologic changes in chick embryo fibroblasts was 70 μg/ml.

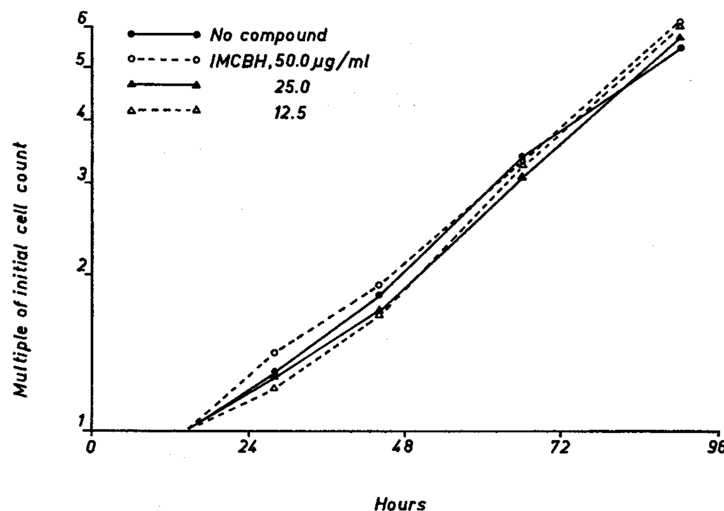

FIG. 2. Lack of inhibitory effect of IMCBH on the multiplication of chick embryo fibroblasts.

The rate of multiplication of chick embryo fibroblasts was studied in the presence or absence of IMCBH. The concentrations of IMCBH employed were 12.5, 25, and 50 μg/ml, which were sufficient to cause marked inhibition of virus release (see below). Plastic Petri dishes were seeded with 6×10^5 cells per dish suspended in 4 ml of growth medium with or without compound. The growth medium consisted of Eagle's MEM containing 10% heat-inactivated fetal bovine serum. Two dishes were used per variable. The cultures were incubated at 37°C in 5% CO_2. At intervals, the supernatant medium was removed, the cell sheets were washed once with PBS deficient in calcium and magnesium, and were trypsinized. The cells were suspended in appropriate volumes of PBS and counted in a hemocytometer. The initial cell count was performed 14 hr after seeding, and all subsequent counts were expressed as a multiple of the initial cell count. There was no obvious difference in the attachment of cells to the plastic surface in the presence or absence of IMCBH. Growth curves of chick

embryo fibroblasts are shown in Fig. 2. As can be seen, IMCBH at concentrations up to 50 μg/ml did not influence the rate of multiplication of the cells.

Minimal Concentration of IMCBH to Inhibit the Multiplication of Vaccinia Virus in Various Cell Cultures.—To determine the minimal concentration of IMCBH to inhibit vaccinia virus multiplication, tube cultures were infected with a small dose of virus and incubated in medium containing increasing concentrations of IMCBH. At intervals, the cultures were examined for development of cytopathic effects (CPE). The experimental procedure was essentially

TABLE I

Minimal Concentration of IMCBH to Inhibit the Development of Cytopathic Effects of Vaccinia Virus in Various Cell Cultures

Cells	Virus strain*	Dose of inoculum	Minimal inhibitory concentration‡
		$TCID_{50}$/tube	*μg/ml*
Chick embryo fibroblasts	P71	3,200	3.1
	MPI	320	25
	IHD	1,000	3.1
Monkey kidney cells	P71	3,200	6.3
	MPI	320	12.5
	IHD	1,000	12.5
L cells	IHD	560	>100
BHK21 cells	P71	630,000§	> 50

* The P71 and MPI strains were passaged in chick embryo fibroblasts. The IHD strain was passaged in L cells.

‡ The lowest concentration inhibiting completely the development of CPE.

§ Titrated in chick embryo fibroblasts.

the same as described by Eggers and Tamm (6). Medium was removed from tube cultures, to each tube 1.9 ml of Eagle's MEM with or without serial 2-fold concentrations of IMCBH was added, and each tube received 0.1 ml of virus. In each experiment, uninfected control cultures with or without serial 2-fold concentrations of the compound were set up, and infectivity titrations of the virus inocula were simultaneously carried out. Three tubes were used per variable. In the experiments with chick embryo fibroblasts, monkey kidney, and L cells, virus inocula of 300–3000 $TCID_{50}$ were used. The cultures were incubated at 37°C in 5% CO_2, and examined daily or every other day for the development of CPE. The final reading was made 5 days after virus inoculation. The lowest concentration of IMCBH which completely inhibited the development of CPE was considered the minimal inhibitory concentration.

In the experiments with BHK21 cells, undiluted virus stock (about 6×10^5 $TCID_{50}$/0.1 ml) was inoculated, the cultures were examined every hour, and the final readings were made 12 hr after inoculation (see below).

Typical results of these experiments are shown in Table I. As can be seen, the development of CPE of vaccinia virus in chick embryo fibroblasts and monkey kidney cells was completely inhibited by relatively low concentrations of

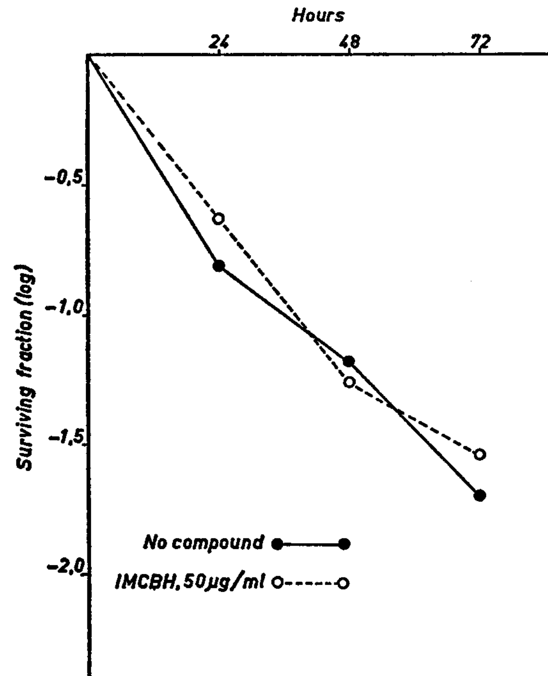

FIG. 3. Lack of direct inactivating effect of IMCBH on the infectivity of vaccinia virus The rate of inactivation of virus at 37°C in the presence or absence of 50 μg/ml of IMCBH was determined.

IMCBH. Of the three virus strains, P71 and IHD exhibited a similar degree of susceptibility in these two cell types. The MPI strain was less susceptible in chick embryo fibroblasts than the other two strains, although it showed a similar susceptibility in monkey kidney cells. These results were consistently and reproducibly obtained in several experiments.

On the other hand, the IHD strain was resistant to IMCBH in L cells. The effect of IMCBH on the multiplication of the P71 and MPI strains in L cells could not be examined because they do not multiply in these cells.

The development of CPE in BHK21 cells infected with large doses of vaccinia virus (P71) was not prevented by IMCBH. It has been found that BHK21 cells

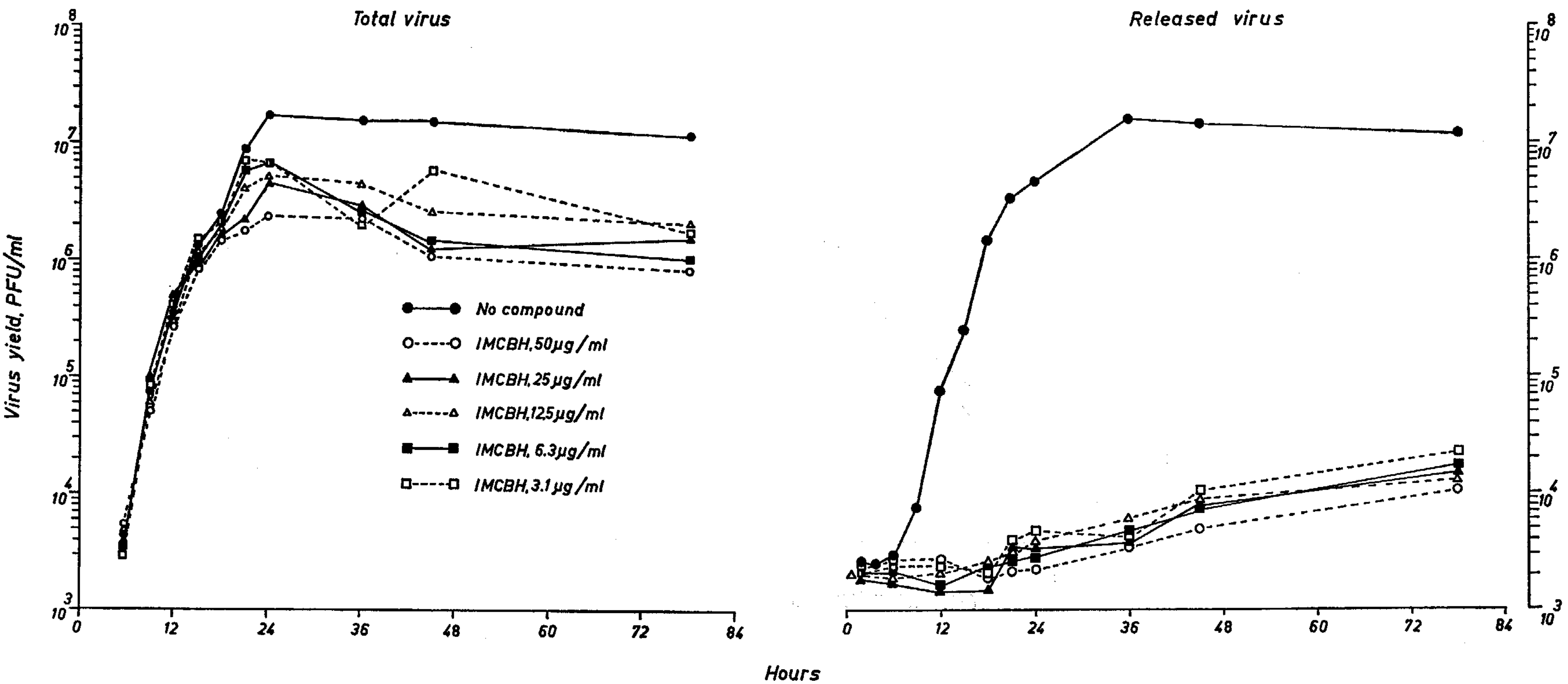

FIG. 4. Effect of IMCBH on vaccinia virus multiplication in chick embryo fibroblasts. The input multiplicity was 1 PFU/cell.

showed definite morphologic changes 3 hr after infection with high concentrations of vaccinia virus (P71) passaged in chick embryo fibroblasts. The changes consisted of cell rounding, agglutination, and later cellular destruction. Almost all cells were affected 7 hr after infection. These changes were not associated with virus multiplication. The cytopathic changes were observed only when large inocula were used, and they no longer occurred in succeeding passages in BHK21 cells. These observations point to the toxic nature of this cytopathic change. In addition, they are in agreement with the findings on the toxic effects of vaccinia virus already reported (7–13).

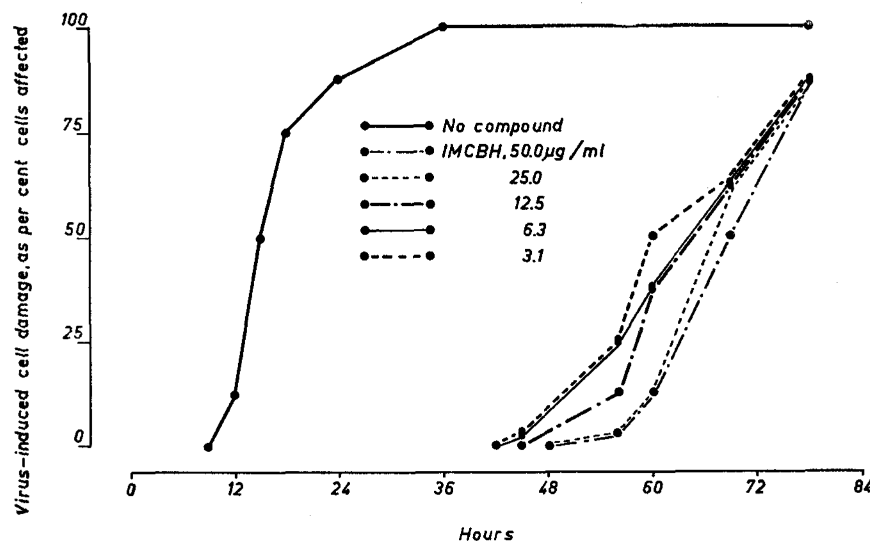

FIG. 5. Effect of IMCBH on the development of cytopathic effects of vaccinia virus in chick embryo fibroblasts. The data are obtained from the experiment shown in Fig. 4.

Lack of Direct Inactivating Effect of IMCBH on Vaccinia Virus.—To examine whether IMCBH has any direct inactivating effect on vaccinia virus, the rate of inactivation of vaccinia virus at 37°C in the presence or absence of 50 μg/ml of IMCBH was determined. To each plastic Petri dish, 4 ml of Eagle's MEM containing about 2×10^5 PFU/ml of vaccinia virus (P71), with or without compound, was added. The dishes were incubated at 37°C. Two dishes per variable were used. At intervals virus material was removed from the dishes and stored at −35°C until assayed by the plaque method. The results are shown in Fig. 3. The rate of decline of infectivity titers at 37°C was similar in the presence and absence of compound. Thus, IMCBH does not cause direct inactivation of vaccinia virus held in contact with concentrations up to 50 μg/ml.

Effect of IMCBH on the Multiplication and Cytopathic Effects of Vaccinia

Virus During a Single Infectious Cycle.—Chick embryo fibroblast cultures were infected with strain P71 at an input multiplicity of 1 PFU/cell. The concentrations of IMCBH used ranged between 3.1 and 50 μg/ml, sufficient to cause marked inhibition of virus multiplication under conditions of infection at low multiplicity (see Table I). The amount of infective virus produced, virus release, and virus-induced cell damage were determined. Cells exhibiting retraction and rounding were considered damaged. Cytopathic changes were expressed as per cent cells affected. The results of a typical experiment are shown in Figs. 4 and 5. The most conspicuous finding in Fig. 4 is a striking difference in the growth curves of total and released virus in IMCBH-treated cultures. The onset and slope of the production of total virus were closely similar in IMCBH-treated and untreated, control cultures. A difference was observed only after 18–21 hr; the growth curves of treated cultures leveled off earlier and the amount of virus produced was about 2.5–7.5 times lower than that in control cultures.

On the other hand, the release of infective virus was markedly inhibited in IMCBH-treated cultures. While in control cultures the phase of logarithmic increase of released virus began 6 hr after virus inoculation, in treated cultures a significant release of virus was first detected 21 hr postvirus inoculation. A steady but only very slight increase of released virus was then observed. 78 hr after virus inoculation, the amount of released virus in treated cultures amounted to about 0.5 to 1% of total virus produced, whereas in control cultures more than 50% had been released.

While cells in control cultures showed a complete CPE 36 hr after virus inoculation, cells in treated cultures exhibited no cytopathic changes up to 42–48 hr postvirus inoculation (Fig. 5). After this time CPE developed, and most cells were affected 78 hr after virus inoculation. The cytopathic changes in treated cultures were microscopically similar to those in control cultures (cell rounding). It should be emphasized that these cytopathic changes in treated cultures were not accompanied by a significant release of virus.

This experiment demonstrates that IMCBH blocks the release of virus from the cells, while intracellular virus multiplication is only slightly affected. This was confirmed in a similar type of experiment, in which total, cell-associated, and released virus was assayed (Fig. 6). In treated cultures, nearly all infective virus remained cell-associated for at least 36 hr, the duration of this experiment. In control cultures, on the other hand, the increase in the amount of released virus occurred at an exponential rate soon after intracellular multiplication was detectable.

Effect of IMCBH Added at Various Times during a Single Cycle of Vaccinia Virus Multiplication.—The following study was carried out to determine the effect of IMCBH on vaccinia virus release and development of cytopathic changes when added at various times during a single infectious cycle. The input multiplicity in these experiments was 1 PFU/cell, and the concentration of

IMCBH was 25 μg/ml. The data in Fig. 7 are from a typical experiment in which IMCBH was added 2, 6, 9, 12, and 15 hr after virus inoculation. The addition of IMCBH as late as 9 hr after virus inoculation prevented approxi-

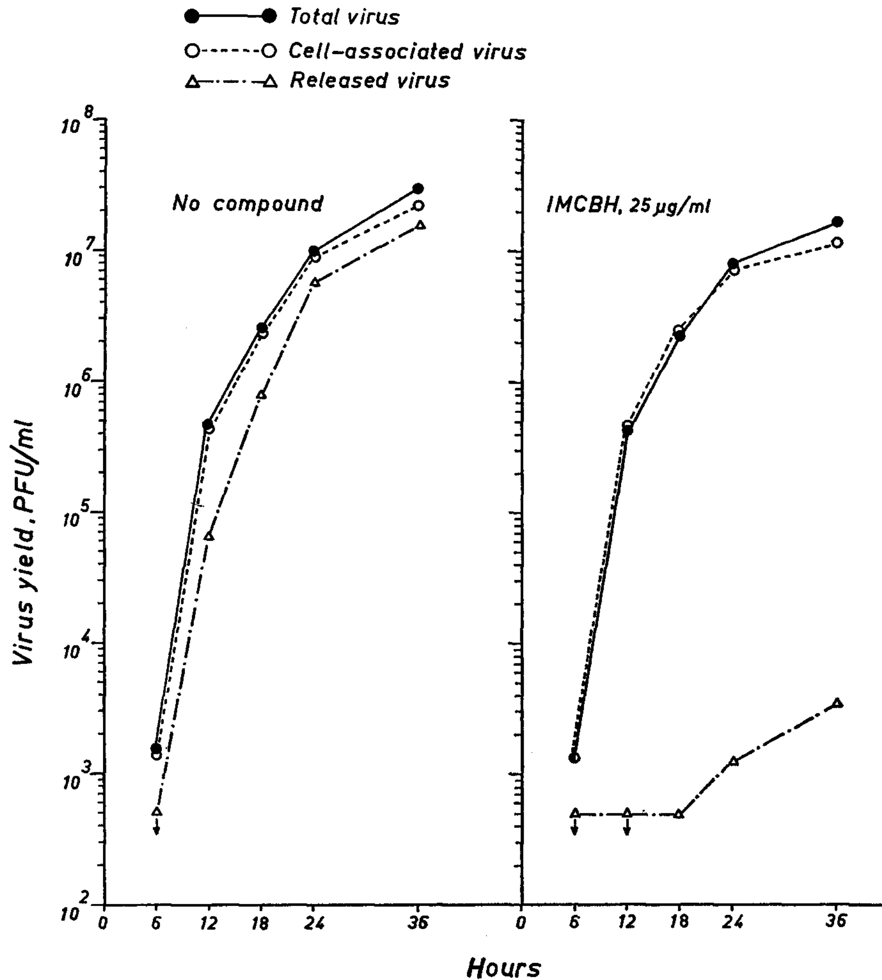

FIG. 6. Effect of IMCBH on the development of total, cell-associated, and released virus during a single infectious cycle of vaccinia virus in chick embryo fibroblasts. The input multiplicity was 1 PFU/cell.

mately 99% of virus release and completely inhibited virus-induced cytopathic changes for the duration of a single cycle experiment. When IMCBH was added at 12 and 15 hr after virus inoculation, at which times virus release proceeded at a logarithmic rate, the addition of IMCBH could still significantly suppress

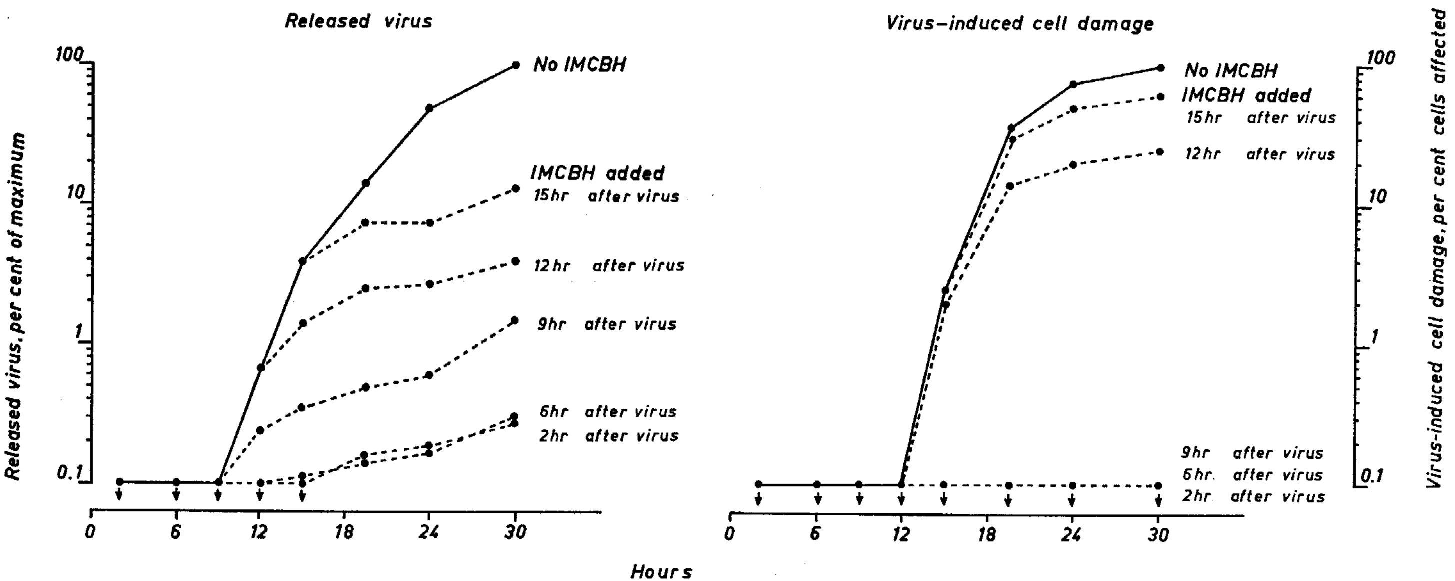

FIG. 7. Effect of IMCBH on virus release and development of cytopathic changes when added at various times during a single infectious cycle of vaccinia virus.

further release of virus. Prevention of CPE in these cases was by far not as marked as inhibition of virus release.

The Virus-Inhibitory Spectrum of IMCBH.—To determine the virus-inhibitory spectrum of IMCBH, the ability of IMCBH to inhibit the multiplication of viruses other than vaccinia virus was investigated. Low doses of the viruses listed in Table II were inoculated into tube cultures of either chick embryo fibroblasts or monkey kidney cells and the development of virus-induced changes was recorded. None of the viruses examined was found to be inhibited by IMCBH at a concentration of 50 μg/ml. Thus, of all viruses examined so far only vaccinia virus was found susceptible to IMCBH.

Lack of Protecting Effect of IMCBH on Vaccinia Virus Infection in Mice and Rabbits.—To determine whether IMCBH affects vaccinia virus infections in

TABLE II

*Viruses Insusceptible to IMCBH**

Cells	Viruses
Chick embryo fibroblasts	Sindbis virus Vesicular stomatitis virus Newcastle disease virus (Miyadera) Fowl plague virus (Rostock)
Monkey kidney cells	Poliovirus type 1 (Mahoney) Echovirus type 12 (Travis) Reovirus type 3 (Dearing) Adenovirus type 7

* Dose of inoculum was 32–320 $TCID_{50}$ per culture. Virus multiplication was not inhibited by IMCBH at 50 μg/ml.

vivo, experiments in mice and rabbits were performed. Mice, weighing 16–20 g, were inoculated intracerebrally with 50 LD_{50} (50% lethal dose) of vaccinia virus (P71). Rabbits were inoculated intracutaneously with 10–1000 LD_{50} (for mice) of the virus. IMCBH was administered five times daily subcutaneously or orally into mice, and orally into rabbits. Maximum doses of IMCBH per day were 400 mg/kg body weight for subcutaneous injection into mice, 800 mg/kg for oral administration into mice, and 500 mg/kg for oral administration into rabbits. The first dose was given 24 hr before virus inoculation. The results of these experiments indicated that IMCBH exhibits no protecting effect on vaccinia virus-infected mice and rabbits.

DISCUSSION

The inhibitory effect of IMCBH on virus multiplication is characterized by two features, i.e., its virus selectivity and its dependence on certain host cells.

IMCBH inhibits only vaccinia virus, but none of a variety of other viruses so far tested, and it is nontoxic to cells at virus inhibitory concentrations. However, while it was found to inhibit vaccinia virus multiplication in chick embryo fibroblasts and monkey kidney cells, it did not do so in L cells. IMCBH did not exhibit any protecting effect on vaccinia virus-infected mice and rabbits. Among other possibilities, such as insufficient concentrations of compound in the target organs, this fact may be due to the dependence of IMCBH action on certain host cells.

The single cycle experiments described indicate that IMCBH, at concentrations ranging from 3.1 to 50 μg/ml, blocks the release of vaccinia virus from chick embryo fibroblasts. It appears that even virus release already under way is inhibitable by IMCBH (Fig. 7). I cannot be excluded, however, that this phenomenon is largely due to asynchrony of infection in the cell population. Intracellular multiplication of vaccinia virus is only slightly inhibited by IMCBH. This finding suggests that the various stages of virus synthesis, i.e., virus adsorption, penetration, uncoating, macromolecular synthesis, and virus assembly remain principally unaffected by IMCBH. IMCBH has no direct inactivating effect on the infectivity of vaccinia virus. It can therefore be concluded that IMCBH is a selective inhibitor of the release of vaccinia virus.

IMCBH inhibits markedly the multiplication of vaccinia virus in cultures infected at low virus/cell multiplicity. This inhibitory effect is undoubtedly due to the protection of a majority of the cells in a culture by the blockade of virus release from primarily infected cells.

As far as we are aware, there are two other reports on inhibition of virus release from infected cells. In 1954, Ackermann and Maassab (14, 15) presented evidence that α-amino-*p*-methoxyphenylmethanesulfonic acid interferes with the release of influenza virus; this compound also affects an early step in virus replication. Becht and Drzeniek (16) recently reported that Congo red inhibits the release of fowl plague and Newcastle disease virus, but there is also a marked inhibition of the formation of hemagglutinin in cells and consequently of cell-associated infective virus.

It is of considerable interest that in IMCBH-treated cultures no cytopathic changes occurred within the span of a single cycle of infection, i.e. 36 hr, whereas in untreated control cultures virus-induced cell damage was closely related in time to the exponential increase phase of virus multiplication. However, also in the treated cultures, extensive cytopathic changes eventually occurred. These ultimately developing cytopathic changes were not accompanied by significant virus release. At present, it cannot be decided whether a causal relationship exists between virus release and virus-induced cell damage. The difficulties arise in part from the fact that similar morphologic alterations may be brought about by different mechanisms. The following findings (N. Kato and H. J. Eggers, unpublished material), however, do not favor the concept that virus release and

development of cytopathic effects are causally related: in monkey kidney cell cultures infected with vaccinia virus and treated with isatin β-thiosemicarbazone, virus formation was inhibited, and consequently virus release could not occur. Nevertheless, the cytopathic changes in cultures treated with isatin β-thiosemicarbazone were marked, while such changes could hardly be detected in cultures treated with IMCBH.

It has been found in the present study that large inocula of vaccinia virus cause early CPE in BHK21 cells without concommitant virus multiplication. Vaccinia virus is known to produce such a toxic CPE in several types of cells (8–13). IMCBH did not inhibit the toxic cytopathic changes induced by vaccinia virus in BHK21 cells. This finding does not necessarily imply that IMCBH has no inhibitory effect on the toxic changes, because the inhibitory action of IMCBH shows host cell dependence, as described above. The studies should be extended to other cells susceptible to the toxic effects of vaccinia virus.

Further investigations on the precise mechanism of action of IMCBH may help to clarify our understanding of the processes involved in release of vaccinia virus.

SUMMARY

N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) is a selective inhibitor of vaccinia virus multiplication. In concentrations up to 50 μg/ml, IMCBH causes neither toxic morphologic changes, nor does it inhibit the multiplication of cells. Viruses other than vaccinia are not affected by IMCBH. The virus-inhibitory effect of IMCBH is dependent on the type of host cell used, i.e., the compound is effective in chick embryo fibroblasts and monkey kidney cells but not in L cells. IMCBH does not exhibit any protecting effect on vaccinia virus-infected mice or rabbits.

IMCBH interferes with virus release: in single cycle experiments in chick embryo fibroblasts, IMCBH strongly blocks the release of vaccinia virus at concentrations as low as 3 μg/ml, while intracellular virus synthesis is hardly affected. Viral cytopathic changes are completely suppressed by IMCBH within the span of a single cycle infection, although extensive changes eventually occur. By inhibiting virus release from initially infected cells, IMCBH markedly inhibits the multiplication of vaccinia virus in cell cultures infected at low virus/cell multiplicities.

IMCBH does not inhibit the early toxic cytopathic changes induced by large inocula of vaccinia virus in BHK21 cells.

We wish to thank Miss Erica Waidner for excellent technical assistance.

BIBLIOGRAPHY

1. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science*. **130:** 432.

2. Dales, S., and R. Kajioka. 1964. The cycle of multiplication of vaccinia virus in Earle's strain L cells. I. Uptake and penetration. *Virology.* **24**:278.
3. Kraus, W., and W. Schäfer. 1963. Apparatur zur Herstellung von primären Kulturen animaler Zellen. *Zentralbl. Veterinärmed.* **10B**:394.
4. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. *Virology.* **26**:100.
5. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**:167.
6. Eggers, H. J., and I. Tamm. 1961. Spectrum and characteristics of the virus inhibitory action of 2-(α-hydroxybenzyl)-benzimidazole. *J. Exp. Med.* **113**:657.
7. Westwood, J. C. N. 1963. Virus pathogenicity. *In* Mechanisms of Virus Infection. W. Smith, editor. Academic Press, New York. 255.
8. Scherer, W. F. 1952. Agglutination of a pure line of mammalian cells (L strain, Earle) by suspensions of vaccinia virus. *Proc. Soc. Exp. Biol. Med.* **80**:598.
9. Brown, A., S. A. Mayyasi, and J. E. Officer. 1959. The agglutination of L cells by vaccinia virus. II. The cell-virus interaction related to the agglutination reaction. *J. Immunol.* **83**:521.
10. Hanafusa, H. 1960. Killing of L-cells by heat and UV-inactivated vaccinia virus. *Biken J.* **3**:191.
11. Nishmi, M., and H. Bernkopf. 1958. The toxic effect of vaccinia virus on leucocytes in vitro. *J. Immunol.* **81**:460.
12. Brown, A., S. A. Mayyasi, and J. E. Officer. 1959. The "toxic" activity of vaccinia virus in tissue culture. *J. Infec. Dis.* **104**:193.
13. Bernkopf, H., M. Nishmi, and A. Rosin. 1959. Effect of active and inactive vaccinia virus preparations on human amnion cell cultures. *J. Immunol.* **83**:635.
14. Ackermann, W. W., and H. F. Maassab. 1954a. Growth characteristics of influenza virus: the influence of a sulfonic acid. *J. Exp. Med.* **99**:105.
15. Ackermann, W. W., and H. F. Maassab. 1954b. Growth characteristics of influenza virus. Biochemical differentiation of stages of development. *J. Exp. Med.* **100**:329.
16. Becht, H., and R. Drzeniek. 1968. The effect of azo dyes on myxovirus neuraminidase and on virus multiplication. *J. Gen. Virol.* **2**:261.