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Reversible Restriction of Vesicular Stomatitis Virus in Permissive Cells Treated with Inhibitors of Prostaglandin Biosynthesis

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Indomethacin, a potent nonsteroidal inhibitor of prostaglandin synthetase (cyclooxygenase) reduced yields of infectious vesicular stomatitis virus in HEp-2 cells more than 99% if added to cultures at levels of 10^{-3} *M* either before or after infection. Other permissive cell lines differed according to the treatment period and drug level required for restricting productive infections. The inhibitory effect of indomethacin was progressively reduced if infection of cells was delayed for increasing times after drug removal. Strong inhibition of viral replication also occurred in cells treated with the cyclooxygenase antagonists naproxen, phenylbutazone, and oxyphenylbutazone whereas phenacetin, which does not block cyclooxygenase function, was inactive. Enhanced viral replication occurred in indomethacin-treated HEp-2 cultures when these cells were subsequently exposed to such substances as prostaglandin E_1, cyclic AMP, or insulin. Conversely, indomethacin-treated cells remained restrictive for VSV if they were subsequently exposed to metabolic inhibitors of functional DNA (actinomycin D or mitomycin C), messenger RNA synthesis (α-amanitin), or protein synthesis (cycloheximide) at concentrations that normally do not compromise viral replication. Pretreatment of HEp-2 cells with mitomycin C markedly shifted the dose response for indomethacin-mediated inhibition of VSV from a 90% inhibitory dose of about 10^{-4} *M* to one of 10^{-9} *M* or lower. These findings suggest that preexisting host factors essential for replication of VSV, although rendered nonfunctional by the drug indomethacin, can be replenished unless their synthesis is blocked by various classes of metabolic inhibitors.

INTRODUCTION

Genetic and biochemical studies have established that the replication of vesicular stomatitis virus (VSV) in permissive cells requires unidentified host factors. Host range or host restricted (*hr*) mutants of VSV previously isolated in this laboratory have been shown to involve impaired virus-specific RNA synthesis in nonpermissive cells and the requirement for multiple host factors based on positive complementation reactions between certain combinations of these mutants (Simpson *et al.*, 1979; Morrongiello and Simpson, 1979). Two of these *hr* mutants were recently shown to involve defective *in vitro* methylation of viral mRNA species (Horikami and Moyer, 1982). Host-dependent temperature-sensitive mutants of VSV with deficient transcriptase function have also been described (Szilagyi and Pringle, 1975).

The inability of the virion-bound RNA polymerase to transcribe complementary genome-length RNA in *in vitro* reactions has provided other systems for demonstrating the essential role of host control elements in the biosynthetic functions of VSV. Full-length complements of genomic RNA have been synthesized *in vitro* with virion nucleocapsids supplemented with crude extracts from infected cells (Batt-Humphries *et al.*, 1979) or in "coupled" transcription-translation systems containing intracellular viral nucleocapsid complexes with or without added VSV mRNAs (Hill *et al.*, 1981; Davis and Wertz, 1982). It is presumed that the latter systems also contained one or more host fac-

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tors that associate with and influence the function of viral replicative complexes.

The dependence of VSV replication on preexisting host factors has been further demonstrated in the present study using nonsteroidal anti-inflammatory inhibitors that are known to compromise prostaglandin biosynthesis, cyclic nucleotides, and various enzyme systems.

MATERIALS AND METHODS

Virus stocks and titrations. Working stocks of VSV-Indiana were grown in BHK-21 cells using reinforced RMEM supplemented with 10% newborn calf serum (NCS) (Simpson *et al.*, 1979). Virus stocks were stored at −80°.

Infectivity titrations of virus stocks or of yields from infected cell lines were done by plaque assay in monolayers of BHK-21 cells using established methods (Simpson *et al.*, 1979).

Cell lines. Continuous lines of HEp-2, HeLa/Ohio, and BHK-21 were maintained with RMEM (10% NCS) in 32-oz glass prescription bottles or 75-cm^2 plastic T-flask cultures as needed. Confluent monolayer cultures in 60-mm plastic dishes were used 1 to 3 days after seeding for drug inhibition experiments or plaque assays. Incubation of cultures was carried out in a humidified atmosphere of 8% CO_2/air in incubators maintained at 37°.

Pretreatment of cells with indomethacin and virus infection. Unless indicated otherwise, the pretreatment of cells with indomethacin was carried out in the presence of RMEM containing 2% newborn calf serum since higher serum levels can abolish or reduce the drug effect observed. After incubation of confluent monolayer cultures with the desired concentrations of indomethacin in RMEM for 24 hr or longer at 37°, the cells were washed with balanced salt solution (BSS) and inoculated with VSV-Indiana at an input multiplicity of 10 PFU/cell or higher. Inocula were adsorbed for 30 min at room temperature after which the cultures were again washed with BSS. Infected cultures were incubated with RMEM lacking serum and frozen at −80° after further incubation at 37° for 24 hr.

Materials and chemicals. All tissue culture media, newborn calf serum, and cycloheximide were purchased from GIBCO Laboratories (Grand Island, N. Y.). Indomethacin, phenacetin, phenylbutazone, aspirin, cyclic adenosine monophosphate (cAMP), prostaglandin E_1 (PGE_1), insulin, and α-amanitin were purchased from Sigma Chemical Company (St. Louis, Mo.). Cholera toxin, actinomycin D, and mitomycin C were purchased from Calbiochem (San Diego, Calif.). Sodium butyrate and sodium fluoride were obtained from Matheson, Cole, and Bell (Norwood, Ohio). Guanosine triphosphate (GTP) was purchased from Boehringer-Mannheim (Indianapolis, Ind.). Naproxen was purchased from Syntex, (Palo Alto, Calif.). Theophyllin and isoproterenol were a gift of Dr. Otto J. Plescia. Oxyphenylbutazone was a gift of the Ciba-Geigy Corporation (Summit, N. J.).

RESULTS

Conditions for inhibition of viral growth by indomethacin. We initially examined the requirements for blocking productive infections of various cell lines with VSV using the antagonist of fatty acid cyclooxygenase, indomethacin. Although this drug can inhibit prostaglandin biosynthesis at concentrations of 10^{-6} *M* or lower (Glass *et al.*, 1977), it was necessary to pretreat permissive HEp-2 cells with 10^{-3} *M* indomethacin in order to reduce yields of VSV to less than 0.1% of yields from untreated control cultures. This inhibitory effect was largely nullified if the culture medium used during either drug treatment or postinfection incubation contained serum at concentrations of 10% or higher. Inglot (1969) also found that serum interferes with the viral inhibitory effect of various antiinflammatory drugs including indomethacin which has a strong binding affinity for blood proteins (Ferreira and Vane, 1975). To avoid this problem, in all subsequent experiments serum was usually omitted from the postinfection medium and used during drug treatment at low concentrations (e.g., 2%) which did not block indomethacin action. It was also important to

use freshly prepared solutions of indomethacin since these stock solutions were found to lose their potency when stored for relatively brief periods. Indomethacin at the concentrations used in this study did not exhibit a virucidal effect (data not shown).

The reversibility of the drug effect was apparent in experiments that involved delayed virus infection after pretreatment of cells with indomethacin and removal of drug. Thus, there was a progressive rise in the amount of infectious virus produced in HEp-2 cells as the time between drug removal and virus infection was increased (Table 1). The restoration of the permissive state for VSV replication was often complete by 24 hr after drug removal, particularly when certain metabolites of the arachadonate cascade were added as described below.

The amount of indomethacin and the treatment period required to render cells highly restrictive for VSV replication differed among various cell lines tested. For example, drug concentrations as low as 10^{-6} *M* produced significant inhibition of VSV in the HeLa/Ohio derivative line by comparison with HEp-2 cells which were less sensitive to such drug levels (Table 2). This differential inhibitory effect was consistently observed when the respective cell lines were tested in independent experiments. BHK-21 cells, which produce relatively high yields of infectious VSV particles (Simpson *et al.*, 1979), were found to be more refractory to the action of indomethacin than other cell lines tested. This property is illustrated in Table 3 where it is seen that a 2-day drug treatment period was required to reduce subsequent yields of VSV in BHK-21 cells by 99% or greater. The differences in drug sensitivity of the cell lines examined could possibly reflect the rate and amount of indomethacin uptake by cells within a given time period.

The above experiments indicated that pretreatment of various permissive cell lines with indomethacin over the concentration range of 10^{-3} to 10^{-6} *M* strongly inhibited the growth of VSV when cells were infected immediately after drug treatment. To determine whether this inhibitory effect occurs exclusively under conditions of pretreating cells with drug,

TABLE 1

RELATIVE YIELD OF VSV IN HEp-2 CELLS INFECTED AT DIFFERENT TIMES AFTER TREATMENT WITH INDOMETHACIN AND REMOVAL OF DRUG[a]

Time of infection after drug removal	24-hr Virus yield (PFU/ml)		Percentage drug inhibition
	Drug treated	Control	
0 time	1.7×10^5	3.9×10^8	99.96
+6 hr	9.3×10^7	6.0×10^8	84.5
+12 hr	2.2×10^8	1.1×10^9	80.0
+24 hr	3.8×10^8	9.5×10^8	60.0
+48 hr	3.2×10^8	3.7×10^8	13.5

[a] Newly established confluent monolayers of HEp-2 cells were incubated for 24 hr at 37° with RMEM-2% NCS either containing or lacking 10^{-3} *M* indomethacin. The cultures were washed with BSS after drug treatment and infected either immediately (0 time) with VSV (m.o.i. = 10) or at the time intervals indicated following additional incubation with RMEM-10% NCS. After virus adsorption, all cultures were washed with BSS and incubated for 24 hr at 37° with medium lacking serum. Clarified culture lysates were titrated for infectious VSV by plaque assay in BHK-21 cells.

TABLE 2

INHIBITION OF VESICULAR STOMATITIS VIRUS IN HEp-2 AND HeLa CELLS TREATED WITH INDOMETHACIN[a]

Drug dose (*M*)	24-hr Virus yield ($\log_{10}$ PFU/ml)			
	HEp-2	% Control	HeLa/Ohio	% Control
None	8.3	100	8.5	100
10^{-3}	5.6	0.19	5.3	0.06
10^{-4}	7.3	10.0	6.6	1.36
10^{-5}	8.0	50.1	7.2	5.10
10^{-6}	ND		7.6	12.6
10^{-7}	ND		8.1	39.8

[a] HEp-2 and HeLa cells ($2\text{-}3 \times 10^6$ cells per 60-mm petri plate) were pretreated with RMEM containing freshly prepared indomethacin and 2% newborn calf serum (NCS) for 24 hr at 37° before infection. Treated cultures were washed with BSS and infected with VSV (m.o.i. = 10). After a 30-min virus adsorption period, the cells were washed and fresh RMEM lacking serum and drug was added. The cultures were incubated at 37° for 24 hr after which they were frozen and virus yields were measured by plaque assay in BHK-21 cells. ND = not determined.

TABLE 3

YIELD OF VSV IN BHK-21 CELLS PRETREATED WITH INDOMETHACIN FOR DIFFERENT TIMES[a]

Condition of treatment		Virus yield at 24 hr	
Time (hr)	Indomethacin concentration	Log_{10} PFU/ml	Percentage control
−6	10^{-3} *M*	9.2	40
	None	9.6	
−24	10^{-3} *M*	8.6	11
	None	9.3	
−48	10^{-3} *M*	6.6	0.4
	None	9.0	

[a] At 6, 24, and 48 hr before virus infection, confluent monolayers of BHK-21 cells were treated with either regular RMEM (2% NCS) or the same medium containing 10^{-3} *M* indomethacin. At 0 time, all cultures were washed with BSS and infected with VSV (m.o.i. = 10). The cultures were incubated at 37° for 24 hr with RMEM lacking serum and drug. Virus yields were determined by plaque assay in BHK-21 cultures.

the yield of VSV in HEp-2 cells treated with indomethacin at different times after infection was measured. Figure 1 shows that 24-hr virus yields were strongly reduced even when drug was added as late as 2 hr after virus infection. These findings indicate that indomethacin exhibits a strong inhibitory effect throughout the entire viral replication cycle of this rhabdovirus.

Viral inhibitory effect of various nonsteroidal anti-inflammatory drugs. Since indomethacin is one of a series of nonsteroidal drugs known for their ability to compromise conversion of arachadonic acid into cyclic endoperoxides (McGiff, 1981), it was of natural interest to test additional drugs of this class for possible inhibitory effects against VSV in permissive cells. Monolayer cultures of HEp-2 cells were treated for 24 hr with each of the six drugs listed in Table 4 and infected with VSV without further drug treatment. Indomethacin was the most potent drug tested although strong inhibition of virus yields was obtained in cells exposed to naproxen, phenylbutazone, and oxyphenylbutazone. Phenacetin is a potent anti-inflammatory drug lacking cyclooxygenase inhibitory activity and its negative activity in this experiment (Table 4) suggests that those drugs showing positive inhibition of viral growth may exert their effect, in part, on functional molecules of prostaglandin synthetase (cyclooxygenase) although other target substances cannot be excluded (see Discussion).

Influence of various modulators of prostaglandins and cyclic nucleotides on the virus inhibitory effect of indomethacin. The synthesis and function of prostaglandins and other metabolites of the arachadonic acid cascade can be selectively modified by various drugs, hormones, and growth factors (McGiff, 1981). We tested a variety of these substances and arachadonate metabolites to determine whether they could alter the inhibitory effect of indomethacin on productive infection of HEp-2 cells with VSV. The first seven substances listed in Table 5 were found to enhance viral rep-

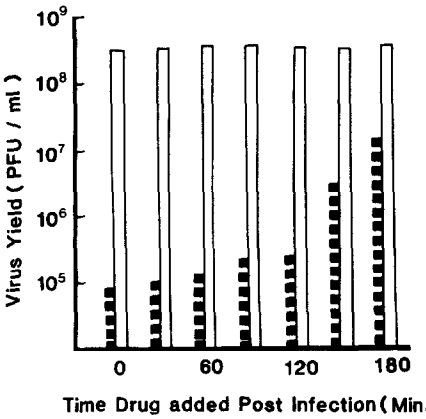

FIG. 1. Comparative yields of vesicular stomatitis virus in HEp-2 cells treated with indomethacin at different times after infection. Confluent monolayer cultures of HEp-2 cells were infected with VSV (m.o.i. = 10). After removing unadsorbed virus by washing with BSS, RMEM lacking serum was added. The cultures were incubated at 37° and at hourly intervals thereafter indomethacin (10^{-3} *M*) was added. The cultures were incubated at 37° for a total period of 24 hr postinfection, and yields of infectious virus were determined by plaque assay in BHK-21 cultures. Virus yields from control cultures (open bars) and cultures treated with indomethacin (broken bars).

TABLE 4

INHIBITION OF VESICULAR STOMATITIS VIRUS REPLICATION IN HEp-2 CELLS TREATED WITH DIFFERENT ANTI-INFLAMMATORY DRUGS [a]

Drug used	Virus yield at 24 hr	
	Log_{10} PFU/ml	Percentage control
None	8.3	100
Indomethacin	5.6	0.2
Naproxen	6.7	2.5
Phenylbutazone	6.8	3.2
Oxyphenylbutazone	6.9	4.0
Aspirin	7.9	40
Phenacetin	8.3	100

[a] HEp-2 cell monolayers were pretreated for 24 hr at 37° with RMEM (2% NCS) containing one of the six drugs listed at a concentration of 10^{-3} *M*. After treatment, all cultures were washed with BSS and inoculated with VSV (m.o.i. = 10). Cultures were washed with BSS after virus adsorption and RMEM lacking serum or drug was added. The 24-hr virus yields from these cultures were titrated by plaque assay in BHK-21 cells.

lication in cells rendered nonpermissive by indomethacin, in which case metabolites such as PGE_1 and cAMP increased virus yields about 150-fold. The substances present in normal serum having this stimulatory effect were not identified although a nonspecific drug-binding effect of serum proteins cannot be excluded. The compounds sodium buyrate, sodium fluoride, and GTP greatly intensified the inhibitory effect of indomethacin resulting in a total block of viral growth. It is likely that such combined treatment causes a general metabolic debilitation of cells rendering them nonpermissive.

Effect of metabolic inhibitors on virus growth in indomethacin-treated cells. The finding that certain prostaglandins, cyclic nucleotides, or modulators of these effector molecules could enhance the recovery of indomethacin-inhibited cells prompted us to study the kinetics of this process under various conditions. Figure 2 illustrates that the progressive increase of virus yields from cells infected at different times after

TABLE 5

RELATIVE YIELDS OF VSV FROM HEp-2 CELLS SEQUENTIALLY TREATED WITH INDOMETHACIN AND VARIOUS MODULATORS OF PROSTAGLANDIN BIOSYNTHESIS [a]

Postinfection treatment	Virus yield (log_{10} PFU/ml) of cultures pretreated with:			
	Indomethacin		No drug	
	Titer	Percentage control	Titer	Percentage control
None	5.61	0.08	8.69	100
Newborn calf serum, 10%	7.81	8.7	8.87	151
PGE_1, 1 μg	7.78	9.4	8.81	132
cAMP, 10 m*M*	7.69	8.3	8.77	120
Insulin, 1.6 μg	7.08	2.7	8.65	92
Theophyllin, 10 μg	6.18	0.17	8.95	181
Cholera toxin, 0.5 μg	6.04	0.17	8.81	132
Isoproterenol, 10 μg	5.9	0.11	8.84	141
Na-butyrate, 10 μg	3.56	0.007	8.74	112
Na-fluoride, 2 μg	<3.18	<0.0003	8.65	92
GTP, 1 m*M*	<3.0	<0.001	7.95	18

[a] HEp-2 cells (2×10^6 cells/60-mm plate) were pretreated with 10^{-3} indomethacin in RMEM (2% NCS) for 24 hr at 37° after which they were infected with VSV (m.o.i. = 10). All cultures were washed with BSS after virus adsorption and incubated for 24 hr at 37° with RMEM containing the substances listed above at the concentrations indicated per milliliter of medium. After incubation, the cultures were frozen and the virus content of clarified cell lysates was measured in BHK-21 cultures by standard plaque assay.

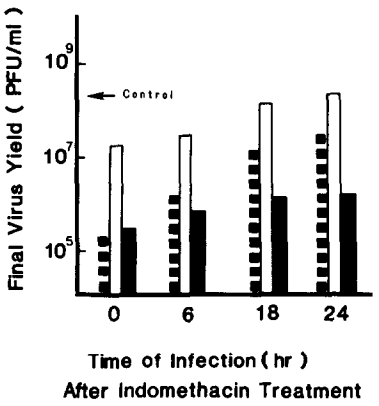

FIG. 2. Reversibility of indomethacin inhibition of VSV replication in HEp-2 cells under conditions of immediate or delayed infection after removal of drug. Confluent HEp-2 monolayers, pretreated for 24 hr with 10^{-3} *M* indomethacin in RMEM (2% NCS), were infected with VSV-Indiana (m.o.i. = 10) immediately after drug treatment or after further incubation at 37° for the time intervals indicated with fresh serum-free medium containing either actinomycin D (0.1 μg/ml), cAMP (1 m*M*) or no supplements. The post-infection medium used for cultures infected immediately after indomethacin treatment (0 time) contained these supplements whereas all other groups received unsupplemented medium. All cultures were incubated for 24 hr at 37° after infection and the virus content of cell lysates obtained by freezing-thawing was measured by plaque assay in BHK-21 cells. The histogram shows virus yields for the different infection groups following post indomethacin incubation with unsupplemented medium (broken bars), medium with cAMP (open bars), and medium with actinomycin D (solid bars). The virus yield from infected control cultures that were not exposed to indomethacin or other drugs was 7.8×10^8 PFU/ml (arrow).

removal of indomethacin is accelerated by cAMP and repressed by low concentrations of actinomycin D. Since the biosynthesis of VSV is not normally inhibited by even higher amounts of actinomycin D, we conclude that the drug effect observed was directed against cellular DNA-dependent RNA synthesis needed for restoration of the viral permissive state. Other inhibitors of functional DNA such as mitomycin C can also block reversal of the indomethacin effect (data not shown).

It was also appropriate to determine whether antagonists of cellular transcription and protein synthesis block the reversal of the indomethacin effect on VSV replication. Indomethacin-treated cells were exposed for different times before infection to the protein synthesis inhibitor, cycloheximide, at concentrations which, when used alone, had no inhibitory effect on growth of virus. Table 6 shows that cycloheximide strongly blocked the reversal of indomethacin inhibition over a 24-hr incubation period based on virus yields that were only 1% of the control. Our use of low concentrations of cycloheximide to obtain this differential effect was suggested by the earlier observation of Cho and Rhim (1979) that such low drug levels can reverse the phenotype of virus-transformed cells without impairing either cell growth or total protein synthesis. It was also found that α-amanitin, a potent inhibitor of RNA polymerase II, partially blocked the recovery of HEp-2 cells from indomethacin inhibition (Table 6). At the concentration used, α-amanitin inhibits the activity of RNA polymerase II in the synthesis of eukaryotic mRNA species but does not impair replication of VSV (Evans and Simpson, 1979).

Effect of mitomycin C on dose response to indomethacin. We considered the possibility that HEp-2 cells might show an increased sensitivity to indomethacin at much lower dose levels if one treated host cells with appropriate metabolic inhibitors *prior to* indomethacin treatment, assuming that the putative target molecules for indomethacin would undergo turnover and depletion under such conditions. Accordingly, we used mitomycin C since this drug can effectively block DNA function at low concentrations without inhibiting growth of VSV (data not shown). Sequential treatment of cells first with mitomycin C (0.1 μg/ml) for 48 hr and thereafter with indomethacin over the dose range of 10^{-3} to 10^{-12} *M* for 24 hr, respectively, markedly increased the indomethacin sensitivity of viral replication (Fig. 3). With this dual treatment regimen, the 90% viral inhibitory dose for indomethacin was shifted from about 10^{-4} to 10^{-9} *M* or lower. This pronounced alteration of the dose response for indomethacin inhibition of VSV in per-

TABLE 6

EFFECT OF INHIBITORS OF CELLULAR MACROMOLECULAR SYNTHESIS ON YIELDS OF INFECTIOUS VIRUS FROM HEp-2 CELLS PRETREATED WITH INDOMETHACIN[a]

Pretreatment of cells with indomethacin	Time of virus infection after indomethacin removal (hr)	24-hr Virus yield (log_{10} PFU/ml) of cultures exposed to:		
		No drug	Cycloheximide	α-amanitin
10^{-3} *M*	0	5.6	5.3	5.7
	+6	6.9	5.0	6.4
	+24	7.9	5.9	6.7
None	0	8.3	8.3	8.3
	+6	8.3	8.4	8.2
	+24	8.4	8.3	8.0

[a] HEp-2 monolayers were incubated at 37° for 24 hr with fresh RMEM (2% NCS) containing or lacking 10^{-3} *M* indomethacin. After this pretreatment, the cultures were washed with BSS and infected with VSV (m.o.i. = 10) either immediately (0 hr) or after further incubation at 37° for 6 or 24 hr with either regular RMEM or medium containing cycloheximide (0.1 μg/ml) or α-amanitin (10 μg/ml). The cultures infected at 0 hr were treated with these drugs during the postinfection incubation period (24 hr at 37°), whereas cultures from the other groups received unsupplemented serum-free medium after infection. The 24-hr virus titer of clarified frozen-thawed culture lysates was determined by plaque assay in BHK-21 cells.

missive cells most likely reflects a reduction of intracellular levels of endogenous host factors which are essential for viral biosynthesis and are products of ongoing cellular metabolism.

We wished to determine whether the total length of time that cells are exposed to mitomycin C correlated with the dose of indomethacin required to inhibit viral replication in HEp-2 cells subjected to dual treatment with these drugs. Concentrations of indomethacin as low as 10^{-6} and 10^{-9} *M*, which normally do not inhibit VSV in these cells (see Table 2), reduced virus yields by 90% or more if mitomycin C was added either before or after the indomethacin treatment had been started (Fig. 4). In this experiment, the total treatment time with indomethacin was 24 hr, whereas the minimum and maximum period that cells were exposed to mitomycin C was 6 and 48 hr, respectively. Thus, even when cells were exposed to mitomycin C only during the last 6 hr of the indomethacin treatment period, there was a significant lowering of the indomethacin dose required to inhibit VSV yields (Fig. 4). These results would suggest that unidentified indomethacin-sensitive host factors in HEp-2 cells which are essential for viral replication are relatively short lived and require functional DNA for their replacement via normal transcriptive processes.

The finding described earlier that BHK-21 cells are more resistant than HEp-2 cells to the inhibitory effect of indomethacin on viral replication whereas HeLa/Ohio cells are relatively more drug sensitive for this property offered the possibility that intracellular levels and turnover rate of drug-sensitive host factors required by VSV might be responsible for the differences observed. We determined the minimum pretreatment period with mitomycin C required to alter the indomethacin dose response of BHK-21 and HeLa/Ohio cells, respectively. Figure 5 illustrates that BHK-21 cells which are relatively resistant to indomethacin required treatment with mitomycin C for at least 24 hr in order to inhibit virus yields by 90% with 10^{-4} *M* indomethacin. By contrast, the highly

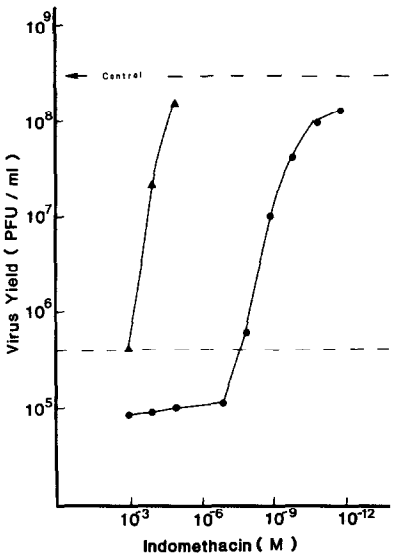

FIG. 3. Modified dose response for inhibition of VSV by indomethacin in HEp-2 cultures resulting from pretreatment of cells with mitomycin C. Subconfluent cultures containing approximately 10^6 HEp-2 cells were incubated at 37° for 48 hr with RMEM (2% NCS) containing or lacking mitomycin C (0.1 μg/ml). After treatment, individual cultures were washed with BSS and incubated for an additional 24 hr with fresh medium containing indomethacin over the concentration range of 10^{-3} to 10^{-12} *M*. Infection of cells with VSV (m.o.i. = 10) was carried out after indomethacin treatment and the cultures were incubated at 37° for 24 hr with unsupplemented serum-free medium. Disrupted cell lysates were measured for virus content by plaque titration in BHK-21 cultures. Virus yields are shown for cells treated with indomethacin only (triangles) and with both mitomycin and indomethacin as described above (circles). Mitomycin C treatment alone had no apparent effect on viral replication and the yield from these control cultures was 4×10^8 PFU/ml (arrow).

indomethacin-sensitive HeLa/Ohio cells showed a more dramatic shift in their indomethacin dose response when pretreated with mitomycin C for only 2 hr, in which case the 90% inhibitory dose of indomethacin was about 10^{-9} *M* instead of about 10^{-6} *M* in the absence of mitomycin C treatment (Table 2, Fig. 5). This result would suggest that these two cell lines differ either for intrinsic levels of indomethacin-sensitive host factors available for viral replication or the rate at which such factors are removed from a functional pool.

DISCUSSION

This study has shown that treatment of cells with indomethacin and certain other nonsteroidal anti-inflammatory drugs renders them nonpermissive for replication of the rhabdovirus, vesicular stomatitis virus. We cannot conclude with certainty whether this inhibition involves specific host factors or a general metabolic impairment of cells reflecting multiple target substrates. That this inhibition does not occur simply at the level of virus adsorption and penetration is indicated by the ability of indomethacin to block viral replication when added to cells following initiation of infection (Fig.

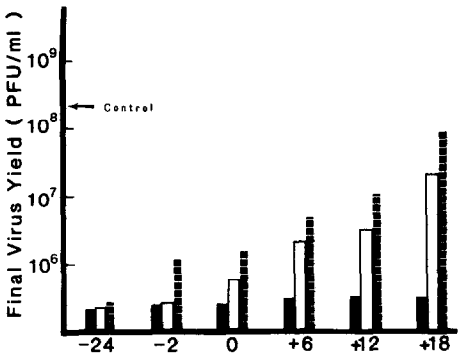

FIG. 4. Time dependence of mitomycin C addition on the alteration of dose response for growth restriction of VSV by indomethacin in permissive cells. Monolayers of HEp-2 cells were exposed to mitomycin C (0.1 μg/ml) in RMEM (2% NCS) at different times either before or after treatment with indomethacin (10^{-3}, 10^{-6}, or 10^{-9} *M*) had been initiated. The total treatment period with indomethacin was 24 hr at 37° after which the cells were washed, infected with VSV (10 PFU/cell) and incubated for 24 hr at 37° with drug-free RMEM lacking serum. Virus infected cultures were disrupted by freezing/thawing and these lysates were titrated for infectious virus by plaque assay in BHK-21 cells. The abscissa shows time of addition of mitomycin C relative to the time that indomethacin was added (0 time) to cells for a 24-hr period. Virus yields from cells treated with indomethacin at 10^{-3} *M* (solid bars), 10^{-6} *M* (open bars), and 10^{-9} *M* (broken bars). The virus yield of cultures treated with mitomycin C only is indicated by the arrow. In the absence of mitomycin C treatment, indomethacin at 10^{-6} and 10^{-9} *M* did not inhibit virus growth (Table 2).

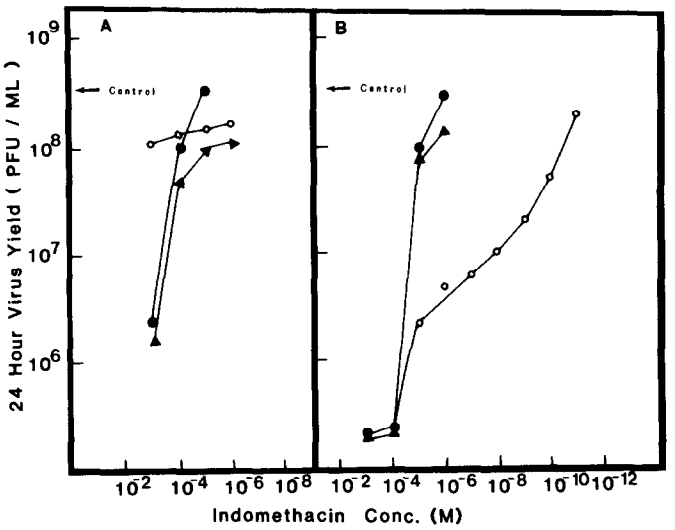

FIG. 5. Relative sensitivity of VSV replication to inhibition by indomethacin in HeLa and BHK-21 cells under conditions of pretreatment with mitomycin C. (A) Monolayer cultures of BHK-21 cells were pretreated with mitomycin C (0.1 μg/ml) for 6, 12, or 24 hr before a 24-hr incubation with RMEM containing different concentrations of indomethacin. The cultures were infected with VSV (m.o.i. = 10) after the indomethacin treatment and further incubated at 37° for 24 hr. Virus yields, determined by plaque assay in BHK-21 cells, are shown for cells pretreated with mitomycin C for 6 hr (open circles), 12 hr (solid circles), or 24 hr (solid triangles), respectively. (B) Monolayers of the HeLa/Ohio cell line were pretreated with mitomycin C for 30, 60, or 120 min before subsequent exposure to indomethacin over the concentration range of 10^{-2} to 10^{-12} *M*. Virus yields are shown for cells pretreated with mitomycin C for 30 min (solid circles), 60 min (solid triangles), and 120 min (open circles), respectively. Virus yields from control cultures treated with mitomycin C only are indicated by the arrows.

1). Of interest is the finding that the permanent cell lines tested varied in their sensitivity to this drug as reflected by the different levels of indomethacin required to suppress production of infectious virus by 90% or higher. This difference is not surprising since it has been recognized that the relative potency of such anti-inflammatory drugs can vary considerably against cell-free enzyme systems such as prostaglandin synthetase (cyclooxygenase) derived from different tissues of various animal species (Flower and Vane, 1974).

Various inhibitory effects of antagonists, modulators, or metabolites of prostaglandin biosynthesis on the replication of animal viruses in cell culture systems have been described. Inglot (1969) reported that addition of indomethacin at 0.05 m*M* to infected cells inhibited one-cycle growth of four different RNA viruses by 90% although quantitative data were lacking. Indomethacin also has been shown to block replication and DNA synthesis of herpes simplex virus in mouse L cells at maximum concentrations similar to those used in this study (Newton, 1979). Conversely, three inhibitors of cyclooxygenase including indomethacin were recently found to block the antiviral effect of interferon in mouse or human cells challenged with VSV without exerting a strong viral inhibitory effect at maximum dose levels of 1 mg per milliliter (Sarkar and Gupta, 1982). Metabolites linked to the arachadonic acid cascade which exert antiviral effects *in vitro* include prostaglandins of the A series (Santoro *et al.*, 1983) and the cyclic nucleotide cAMP (Robbins and Rapp, 1980). No unifying concept has emerged from such studies regarding the possible mode of action of these agents against DNA or RNA animal viruses.

The viral inhibitory concentrations of indomethacin initially used in this study greatly exceed amounts that are necessary to compromise prostaglandin biosynthesis initiated by the enzyme cyclooxygenase (Glass *et al.*, 1977). Protein kinase activity *in vitro* is also blocked by these high drug levels (Goueli and Ahmed, 1980) whereas much lower concentrations of indomethacin are also inhibitory for several other enzyme systems including phosphodiesterase (Flower and Vane, 1974), presumably owing to the affinity of this anti-inflammatory drug for lipophilic peptide sequences (Humes *et al.*, 1981). Although multiple targets apparently exist for indomethacin, some of the findings obtained in this study suggest that the inhibition of VSV by this drug involves one or more elements of the prostaglandin biosynthetic pathway. First, other potent antagonists of fatty acid cyclooxygenase such as phenylbutazone exert a strong antiviral effect in our test system whereas phenacetin, an anti-inflammatory drug not active against cyclooxygenase, was inactive in suppressing VSV infections (Table 4). Second, exogenous addition of PGE_1 or cAMP to infected cells following indomethacin treatment can substantially reduce the inhibitory effect of this drug, suggesting that the metabolic block imposed can be bypassed in this manner. The recovery of the permissive state for viral replication following removal of indomethacin is also significantly accelerated by such physiological effectors and both cAMP and serum are known to stimulate prostaglandin biosynthesis in cultured mammalian cells (Samuelsson *et al.*, 1978). Substances such as theophyllin or isoproterenol which increase intracellular cAMP levels were also found to counteract the inhibitory effect of indomethacin seen in this study. Thus, this cyclic nucleotide may play a critical role in the drug-mediated inhibition observed.

Various metabolic inhibitors of cellular macromolecular synthesis were shown to modify both the sensitivity of cultured cells to inhibition by indomethacin and the recovery of these cells after drug treatment based on their permissiveness for viral replication. The ability of relatively low concentrations of actinomycin D to retard the progressive reversal of indomethacin inhibition in HEp-2 cells suggests that active cellular transcription is required for replacement of labile host factors that are compromised by indomethacin treatment. Further support of this concept comes from the finding that α-amanitin, the inhibitor of RNA polymerase II, exerts a similar effect on the recovery of indomethacin-inhibited cells. Since both actinomycin D and α-amanitin do not normally impair VSV biosynthesis, the effects obtained with these drugs indicate that active cellular transcription is an important event in the recovery of cells from indomethacin inhibition. The protein synthesis inhibitor, cycloheximide, at low concentrations that do not adversely affect VSV replication also strongly interferes with restoration of the viral permissive state after treatment of cells with indomethacin. Cycloheximide and actinomycin D may prevent reversal of the indomethacin effect by directly interfering with prostaglandin biosynthesis since Pong *et al.* (1977) have shown that these drugs block release of membrane-bound archadonate as a substrate for cyclooxygenase.

Mitomycin C was used to demonstrate that this antagonist of functional host cell DNA markedly increases the viral inhibitory effect of indomethacin at submicromolar concentrations (Fig. 3). Indomethacin at 10^{-9} *M* concentration becomes inhibitory for viral replication in HEp-2 cells or HeLa cells under appropriate conditions of treatment with mitomycin C, suggesting that indomethacin may be acting on cyclooxygenase-dependent prostaglandin biosynthesis rather than on other enzyme systems which are known to be sensitive to indomethacin at relatively higher drug concentrations (Flower and Vane, 1974; Goueli and Ahmed, 1980). The observation that BHK-21 cells require a more prolonged treatment with mitomycin C than HeLa/Ohio cells to affect a significant shift in their indomethacin dose response, could signify that the indomethacin-sensitive host factors involved are either more abundant in BHK-21 cells or

have a lower binding affinity for this drug as an allosteric inhibitor.

Work near completion indicates that indomethacin severely affects VSV biosynthesis at the level of RNA transcription, replication, and protein synthesis. Our aim is to identify the host factors compromised by such drugs and understand their possible role in the viral replication cycle. Prostaglandins and other physiological effectors influenced by the arachadonic acid cascade may ultimately be recognized as important biological regulatory agents for productive infections with VSV and other animal viruses.

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