Selective Irreversible Inactivation of Replicating Mengovirus by Nucleoside Analogues: a New Form of Viral Interference

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We describe the selective irreversible inhibition of mengovirus growth in cultured cells by a combination of two pyrrolopyrimidine nucleoside analogues, 5-bromotubercidin (BrTu) and tubercidin (Tu). At a concentration of 5 \(\mu\)g/ml, BrTu reversibly blocked the synthesis of cellular mRNA and rRNA but did not inhibit either mengovirus RNA synthesis or multiplication. BrTu is a potent inhibitor of adenosine kinase, and low concentrations of BrTu (e.g., 0.5 \(\mu\)g/ml), which did not by themselves inhibit cell growth, blocked phosphorylation of Tu and thus protected uninfected cells against irreversible cytotoxicity resulting from Tu incorporation into nucleic acids. In contrast, in mengovirus-infected cells, BrTu did not completely inhibit Tu incorporation into mengovirus RNA, allowing the formation of Tu-containing functionally defective polynucleotides that aborted the virus development cycle. This increased incorporation of Tu coupled to mengovirus infection could be attributed either to a reduction in the inhibitory action of BrTu and/or its nucleotide derivatives at the level of nucleoside and nucleotide kinases and/or, perhaps, to an effect upon the nucleoside transport system. The virus life cycle in nucleoside-treated cells progressed to the point of synthesis of negative strands and probably to the production of a few defective new positive strands. Irreversible virus growth arrest was achieved if the nucleoside mixture of BrTu (0.5 to 10 \(\mu\)g/ml) and Tu (1 to 20 \(\mu\)g/ml) was added no later than 30 min after virus infection and maintained for periods of 2 to 8 h. The cultures thus “cured” of mengovirus infection could be maintained and transferred for several weeks, during which they neither produced detectable virus nor showed a visible cytopathic effect; however, the infected and cured cells themselves, while metabolically viable, were permanently impaired in RNA synthesis and unable to divide. Although completely resistant to superinfecting picornaviruses, they retained the ability to support the growth of several other viruses (vaccinia virus, reovirus, and vesicular stomatitis virus), showing that cured cells had, in general, retained the metabolic and structural machinery needed for virus production. The resistance of cured cells to superinfection with picornaviruses seemed attributable neither to interferon action nor to destruction or blockade of virus receptors but more likely to the consumption of some host factor(s) involved in the expression of early viral functions during the original infection.

The compound 5-bromotubercidin (BrTu) (Fig. 1) is a cytotoxic pyrrolopyrimidine analogue of adenosine. The metabolism of this compound and its effects on nucleic acid synthesis in cultures of mouse and of chicken embryo fibroblasts have been reported elsewhere (11, 12). For purposes of the present work, the following properties of BrTu are recalled: (i) BrTu inhibits fibroblast growth and RNA synthesis, and these effects are fully reversible (6); (ii) BrTu inhibits the synthesis of high-molecular-weight cellular RNA species (i.e., mRNA and rRNA) but does not inhibit either mengovirus RNA synthesis or multiplication; and (iii) BrTu enters the cellular nucleotide pool by conversion to the 5’-monophosphate and is thus a substrate for adenosine kinase. However, as shown below, BrTu is, like 5-iodotubercidin (21, 46), also a potent inhibitor of adenosine kinase, and, as such, can be used to modulate the cellular uptake of other, more cytotoxic adenosine analogues, such as tubercidin (Tu) (1), 7-deazanebularin (DN) (13), toycamycin, formycin, and 8-aminoadenosine (12). The possibility of controlling the rate of analogue metabolism in this way prompts us to differentiate between viral and host replicating systems, since studies of the respective polymerases in cell-free systems have shown clear-cut differences in both affinity for and incorporation of nucleotide analogues (29). With these considerations in mind, we have studied the effects of analogue mixtures in an experimental model cell culture system in which mouse fibroblasts (strain L-2) act as the host for the virulent RNA virus mengovirus. The results presented here reveal that appropriate combinations of nucleoside analogues can selectively and irreversibly block viral RNA synthesis and thereby interrupt the virus life cycle under conditions that do not affect the viability of normal uninfected host cells. In this model system, therefore, infected cultures may be “cured” of mengovirus infection. The treated or cured cells, in which mengovirus is prevented from replicating, show several unusual properties, including a novel pattern of immunity to superinfection by picornaviruses.

MATERIALS AND METHODS

Cells and viruses. All experiments were performed by using monolayer cultures of L-2 and HeLa cells grown in Eagle’s minimal medium supplemented with 5% (vol/vol) fetal bovine serum; chicken embryo fibroblasts were grown in Dulbecco’s modified Eagle’s medium containing 10% (vol/vol) fetal bovine serum (all from GibcoBRL Life Technologies, Gaithersburg, Md.). The procedures used for maintenance and propagation of cell cultures, for establishment of rates of cell growth, for virus infections and titration, and for
monitoring of the incorporation of radioactive precursors into macromolecules have been described in detail elsewhere (11, 13).

The strains of mengovirus and vaccinia virus were the same as those used in previous work (13). Reovirus type 3, Sindbis virus, vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) were obtained from P. Gomatos, P. Choplin, and S. Silverstein, respectively.

Antisera. Antisera were prepared by injecting rats with increasing concentrations of purified mengovirus in sterile phosphate-buffered saline (1 x 10^9 to 5 x 10^9 PFU) over a 4-week period. A week after the last injection, the rats were exsanguinated and serum was separated from blood cells in the presence of 2% (wt/vol) sodium citrate. Antisera to reovirus 3 were prepared by injecting rabbits with increasing concentrations of purified virus (1 x 10^8 to 5 x 10^8 PFU) in the same way as described for mengovirus.

Preparation of mengovirus double-stranded RNA. Mengovirus double-stranded RNA was prepared by the method of Bishop and Koch (8). Briefly, the cytoplasmic RNA from mengovirus-infected, actinomycin-treated cells was prepared by phenol extraction and ethanol precipitation. After ethanol precipitation, the RNA was dissolved in 0.02 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.1% sodium dodecyl sulfate, and layered over a linear 15 to 30% sucrose gradient in the same buffer. Gradients were centrifuged at 22°C for 16 h at 25,000 rpm (Beckman SW25.1 rotor), 0.5-ml fractions were collected, and the A_{260} of each fraction was determined. RNase resistance was determined by diluting 50 μl of each fraction with an equal volume of 0.6 M NaCl-0.06 M sodium citrate and digesting it for 30 min at 25°C with 100 μg of pancreatic RNase per ml. All fractions were then precipitated with trichloroacetic acid and counted as previously described (13). The fractions containing RNase-resistant mengovirus double-stranded RNA were pooled, and they corresponded to the 18S-to-20S peak; mengovirus single-stranded RNA sediments faster than double-stranded RNA. The fractions corresponding to single-stranded or double-stranded mengovirus RNA were pooled, dialyzed against 1x SSC (0.15 M NaCl plus 0.015 M sodium citrate), and annealed to mengovirus standard RNA.

Preparation of nuclear and cytoplasmic RNAs. RNAs were extracted from nuclear and cytoplasmic fractions and analyzed by polyacrylamide gel electrophoresis as previously described (11).

Preparation of mengovirus and poliovirus RNAs. The procedure used for extraction of viral RNA from purified virions (13) of mengovirus and poliovirus was that of Scherrer and Darnell (40), except that dithiothreitol was added prior to phenol extraction.

Hybridization experiments. Annealing of mengovirus RNA extracted from purified virions to actinomycin-resistant mengovirus single-stranded or double-stranded RNA extracted from virus-infected cells was carried out as previously reported (17).

Labeling of nucleoside analogues. Nucleoside analogues were generously provided by H. Wood and R. Engle, Drug Development Branch, National Cancer Institute, Bethesda, Md., and L. Townsend, Department of Chemistry, University of Utah. [3H]Tu and [3H]BrTu were prepared at New England Nuclear Corp. by catalytic exchange with tritium (45); [3H]Tu was generously provided by G. Acs, Mt. Sinai Medical Center, New York, N.Y. The preparation of [3H]DN has been reported previously (13). All other radioactive precursors were purchased from regular commercial suppliers.

RESULTS

BrTu protects cells against other cytotoxic adenosine analogues. The observations that form the basis for all subsequent experiments are represented in Fig. 2 and 3. From data presented elsewhere (11, 12), it is known that BrTu reversibly inhibits the proliferation of L cells; complete growth stoppage requires 5 μg of BrTu per ml (15 μM), whereas lower drug concentrations produce correspondingly less inhibition. At a BrTu concentration of 0.5 μg/ml, a consistent rate of cell multiplication was maintained, although it was not quite as rapid as in untreated control cultures (Fig. 2).

In contrast to BrTu, whose effects on cell growth are reversible, Tu is both more potent and, at concentrations above 0.01 μg/ml (37 nM), rapidly and irreversibly cytotoxic. As seen in Fig. 2, the complete arrest of cell multiplication that occurred with Tu at 0.1 μg/ml (0.37 μM) was prevented upon addition of small supplements of BrTu. Thus, the combination of BrTu at 0.5 μg/ml and Tu at 1.0 μg/ml, growth proceeded at essentially the same rate as in BrTu alone. With BrTu at 1.0 μg/ml, growth was resistant to as much as 5 μg of Tu per ml, a concentration much higher than that required to produce irreversible loss of cell viability by Tu alone.

Figure 3 illustrates the protective effect of BrTu against cell killing by DN under somewhat different conditions. Here the cultures were exposed for a short period to the indicated concentrations of DN or BrTu alone or to mixtures of the two, and their subsequent growth rate in drug-free medium was measured. The growth of all BrTu-treated cultures, even those simultaneously exposed to DN, was the same as that of the untreated control, whereas cell multiplication did not resume in the culture that had been transiently incubated with DN alone. Identical results were obtained with Tu in comparable experiments, and entirely similar protective effects of BrTu were observed with other irreversibly cytotoxic nucleosides, such as toyocamycin or 8-aminoadenosine (data not shown).

In short, the presence of BrTu simply nullified the cytotoxic action of the other adenosine analogues.

BrTu blocks uptake of other adenosine analogues. The results of several experiments demonstrated that this protective
action of BrTu was associated with a great reduction, or a complete block, of cellular uptake of other cytotoxic nucleosides, such as Tu and DN. Thus, the incorporation of \(^{3}H\)Tu (Fig. 4) or \(^{3}H\)DN (data not shown) into DNA, RNA, and the acid-soluble nucleotide pool of L cells was reduced to almost undetectable levels in the presence of BrTu; by inhibiting adenosine kinase (12, 21, 22), BrTu prevents phosphorylation and thus blocks uptake and incorporation of Tu and DN, providing a means for modulating the metabolism of these cytotoxic compounds in a controlled fashion and prompting a search for conditions under which their toxic properties might be directed in a selectively antiviral way. This perspective was further encouraged by (i) previous ultrastructure (2, 10) and ion permeability studies (23, 30, 31) suggesting that plasma membrane modifications were associated with virus infection, (ii) by reports (29) that viral and cellular polymerases differed in both substrate specificity and affinity (\(K_m\)) for nucleoside triphosphates, and (iii) by the possibility that viral and cellular polymerases might draw on different intracellular nucleotide pools (24, 32, 37).

With these considerations in mind, and given the toxicity that accompanied the incorporation of even small amounts of Tu into RNA, it appeared possible that antiviral selectivity might be achieved by promoting some Tu incorporation into infected cells while preventing uptake into uninfected cells. A range of BrTu-Tu ratios and concentrations was assessed to identify conditions that might yield the desired pattern of \(^{3}H\)Tu incorporation; several combinations ultimately proved effective for blocking mengovirus growth without permanently damaging uninfected cells. For example, exposure of normal L cells to a combination of BrTu plus Tu (10:20 \(\mu\)g/ml or 1:2 \(\mu\)g/ml) for as long as 12 h left no impairment of cell viability after drug removal (data not shown)—nucleic acid synthesis was inhibited during incubation with the analogues, but it resumed and normal growth was reinitiated when the drugs were removed and replaced with fresh medium.

To compare the incorporation of \(^{3}H\)Tu under these conditions in normal and mengovirus-infected cells, we performed the experiments outlined in Fig. 5A. Several cell cultures were infected with mengovirus (curves 1 to 3), and another served as an uninfected control (curve 4). Two cultures (curves 1 and 3) were pretreated with actinomycin (2 \(\mu\)g/ml) for 20 min prior to infection, and in addition, three cultures (curves 2, 3, and 4) were supplemented with BrTu. As seen in curve 1, in the absence of BrTu, large amounts of \(^{3}H\)Tu were incorporated into RNA beginning 3 h after infection. This incorporation was actinomycin resistant and therefore virus specific, confirming that Tu is a substrate for mengovirus RNA synthesis (1). Comparison of curves 2 and 4 shows that whereas \(^{3}H\)Tu incorporation into cellular nucleic acids was almost totally blocked by BrTu (curve 4), incorporation in companion infected cultures (curve 2) was significantly higher. This increased incorporation was only minimally reduced by actinomycin (curve 3) and was therefore essentially virus directed. Thus, the presence of BrTu protected uninfected cultures (curve 4) but not infected cultures (curves 2 and 3) against Tu incorporation. Since incorporation of Tu into virus-specific polynucleotides blocks the yield of infectious progeny (1) and also prevents the development of a microscopically observable cytopathic effect (unpublished observation), it seemed that selective arrest of virus growth was achievable without damage to uninfected cells. Further, identical results were obtained in the absence of actinomycin, thereby excluding any involvement of this antibiotic in the phenomenon. We concluded that combined treatment with BrTu and Tu early in the infectious cycle irreversibly aborts virus multiplication and cures the fibroblasts of the
TABLE 1. Mengovirus growth is blocked irreversibly after exposure to BrTu and Tu*  

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Time of drug addition (min) postinfection</th>
<th>No. of PFU/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (0-h control)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>2 (24-h control)</td>
<td>650</td>
<td></td>
</tr>
<tr>
<td>3 (BrTu + Tu)</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>4 (BrTu + Tu)</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>5 (BrTu + Tu)</td>
<td>3.74</td>
<td></td>
</tr>
<tr>
<td>6 (BrTu + Tu)</td>
<td>3.25</td>
<td></td>
</tr>
<tr>
<td>7 (BrTu + Tu)</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>8 (BrTu + Tu)</td>
<td>13.8</td>
<td></td>
</tr>
</tbody>
</table>

* L2-cell monolayers (2 × 10^5 cells per 60-mm-diameter petri dish) were infected with mengovirus at a MOI of 10. After adsorption (1 h, 37°C), the medium was removed and residual unadsorbed virus was neutralized by incubation for 25 min at 37°C in medium containing rat anti-mengovirus serum. The antiserum was then removed, the monolayers were washed, fresh medium was added, and the cultures were incubated for the indicated periods before addition of BrTu (10 μg/ml) and Tu (20 μg/ml). Except for culture 8, in which the drugs were maintained continuously after addition, all of the cultures were exposed to the drugs for 8 h, following which they were washed and incubated in drug-free medium for a further 24 h. The cultures were then collected, and after three cycles of freezing and thawing, the virus titer was determined as described in reference 13.

infecting virus. Additional experiments performed to characterize this result were as follows.

(i) The exposure of the culture to BrTu plus Tu was varied by changing the time of addition, the length of incubation, and the ratio of the two nucleosides. To accomplish the cure, the nucleoside mixture could be added at any time before the infecting virus but, in any case, no later than 30 min after infection; some degree of resistance to the analogues developed progressively thereafter, and a variable but significant number of viral progeny then appeared at some time following drug removal (Table 1). Provided that the postinfection time limit of 30 min was observed, the cultures could be cured by exposure to the nucleosides for periods of 2 to 8 h (Table 2). The concentrations of the two analogues could be varied over the following ranges with no change in response: BrTu, 1 to 20 μg/ml; Tu, 0.5 to 20 μg/ml. The nucleosides did not interfere with virus adsorption, penetration, or uncoating (vide infra), and virus multiplication could not be blocked merely by transient pretreatment of uninfected cells.

(ii) Since all of the previous experiments were performed at a high multiplicity of infection (MOI), curing experiments were repeated at a low MOI as a more rigorous test of the completeness and irreversibility of the analogue-induced block in infecting virus.
TABLE 2. Effect of duration of treatment with BrTu plus Tu on mengovirus multiplication

<table>
<thead>
<tr>
<th>Treatment (No. of medium containing drugs added)</th>
<th>Duration of treatment (h)</th>
<th>No. of PFU/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 h)</td>
<td></td>
<td>719</td>
</tr>
<tr>
<td>Control (24 h)</td>
<td></td>
<td>2.06</td>
</tr>
<tr>
<td>BrTu + Tu</td>
<td>2</td>
<td>0.62</td>
</tr>
<tr>
<td>BrTu + Tu</td>
<td>4</td>
<td>0.50</td>
</tr>
<tr>
<td>BrTu + Tu</td>
<td>8</td>
<td>0.03</td>
</tr>
<tr>
<td>BrTu + Tu</td>
<td>Continuous</td>
<td>0.006</td>
</tr>
</tbody>
</table>

* L-2-cell monolayers (2 × 10⁵ cells per 60-mm-diameter petri dish) were pretreated as indicated for 30 min with a mixture of BrTu (10 μg/ml) and Tu (20 μg/ml) and then infected with mengovirus at an MOI of 10. After virus adsorption (1 h, 37°C), unadsorbed virus was removed as described in Table 1, footnote a, and fresh medium containing drugs was added. Following incubation for 2, 4, or 8 h, the cells were washed free of the drugs and incubation was continued for a further 24 h in drug-free medium; the cultures were then harvested, and the virus titer was determined as described in Table 1, footnote a.

Properties of cured cells: morphology, survival, and macromolecule synthesis. It appeared desirable to study some of the properties of infected and cured fibroblasts to identify any changes that might shed light on some early viral functions. The cured cells appeared morphologically intact and showed no cytopathic effect or other evidence of virus infection (data not shown). However, while they failed to produce viral RNA or viral progeny, the cured cells had lost the ability to divide (Fig. 6). Except for the loss of visible nucleoli, some increase in size, and slight vacuolization, their morphology was essentially unaltered for at least 3 days; during this period, they excluded trypan blue, remained attached to the surface of the petri dish (Fig. 6), and maintained a variety of synthetic activities. After 3 to 4 days, pyknotic nuclear changes began to appear and the cells slowly but progressively detached from the monolayer. Hence, although the nucleosides completely prevented virus multiplication, they did not nullify virus-induced loss of cell division.

Cure permits observation of early events in infection in a background that is uncomplicated by virus growth. The irreversible loss of reproductive viability prompted us to monitor macromolecule synthesis in cured cultures. As seen in Fig. 7A to C, there was a significant reduction in the three major types of macromolecule synthesis. Although DNA replication decreased compared with that of the uninfected control, it continued at a progressively slower rate for some hours following infection and cure. RNA synthesis was also lower in cured cultures than in uninfected controls, and it appeared likely that this decrease was responsible for the concurrent drop in protein synthesis (Fig. 7C).

We compared the pattern of RNA synthesis in cured cultures with those of (i) nucleoside-treated but uninfected controls and (ii) untreated, uninfected controls by using double labeling with [³²P] and [³H]uridine. The combination of BrTu plus Tu (10 and 20 μg/ml, respectively) reduced the cytoplasmic RNA labeling profile (28S, 18S, and 4S) of uninfected cells transiently; this effect (data not shown and reference 11) was reversed to near normal within 2 h, and fully normal rates and patterns of synthesis were restored by 8 h following removal of the drugs. The pattern in cured cultures (from which the nucleosides had been removed) was quite different: there was virtually no cytoplasmic label in 28S and 18S, and 4S labeling was significantly decreased (data not shown). The respective nuclear RNA patterns showed that the labeling of newly formed 45S RNA was reduced by more than two-thirds in the infected-and-cured cultures compared to that in the uninfected controls. Thus, despite its replicative inactivation by BrTu plus Tu, the virus had greatly reduced the rate of rRNA precursor synthesis and, in addition, completely suppressed the conversion of precursor to 28S and 18S cytoplasmic RNAs.

These findings did not establish how far the mengovirus life cycle had progressed before it was blocked by the nucleoside treatment, and it was of interest to define the state of the infecting nucleic acid and the extent of replication, if any. The small amount of newly synthesized virus-specific RNA formed...
In the presence of the analogues (Fig. 5) provided material permitting limited characterization.

A large-scale culture was pretreated with actinomycin, infected and maintained in the presence of BrTu plus Tu, and labeled with [3H]uridine; the RNA was extracted and analyzed by centrifugation on sucrose gradients. The radioactivity profiles of both single- and double-stranded RNAs resembled those previously reported (9) for picornavirus infection, the main peak sedimenting at a position expected from newly synthesized viral RNA in this and other systems (4, 5). The results (not shown) established that in the presence of BrTu plus Tu, the virus life cycle had progressed at least to the point of negative-strand synthesis required for double-stranded RNA formation and, probably, to the production as well of some new positive strands; the latter were present as single-stranded molecules and accounted for the radioactivity in the more rapidly sedimenting portion of the profile. The production of negative strands was rigorously demonstrated by annealing to viral RNA, but the synthesis of positive strands was not unambiguously demonstrated.

Superinfection of cured cells. The cure of mengovirus infection yielded cells that were metabolically viable, impaired in RNA synthesis, and unable to divide, and their lengthy survival made it possible to assess their ability to support viral growth. Accordingly, we measured the multiplication of one DNA virus—vaccinia virus—and three RNA viruses (reovirus, VSV, and mengovirus) in freshly cured cultures. With the exception of mengovirus, these viruses grew to high yields (Table 4), showing that cured cells had retained all of the elements needed for virus production.

The block to superinfection with mengovirus was unexpected, and we explored a variety of conditions that might have facilitated reinfection. These included (i) an increase in the MOI to the level of 75 PFU/cell, (ii) prolonged periods of incubation with high levels of superinfecting virus, and (iii) exposure to viral RNA under conditions that led to productive infection in control cultures (data not shown). None of these promoted the reinfection of mengovirus growth, suggesting that the block to superinfection was not based on virus uptake or uncoating but rather involved some early intracytoplasmic cellular or viral function.

To establish whether the cure phenomenon and resistance to superinfection were fortuitous events restricted to a single host-virus system or potentially of more general significance, we studied the effects of BrTu plus Tu on mengovirus growth in HeLa cells because this cell line, unlike L cells, also supports the growth of several other picornaviruses, including poliovirus. As seen in Table 5, HeLa cells could be cured of either mengovirus or poliovirus infection by exposure to BrTu plus Tu just as L cells could. Further, in each case, the cured cultures were resistant to superinfection both by the originally infecting virus and by the heterologous picornavirus. Hence, the interference observed in cured cultures exposes both a viral function and a viral requirement for one or more cellular functions that are common to at least two picornaviruses.

Effect of BrTu and Tu on other viruses. We surveyed a spectrum of different animal viruses to establish whether the analogue-induced cure might be applied to viruses other than picornaviruses; all gave negative results (Table 6). The growth of NDV, Sindbis virus, and vaccinia virus was profoundly inhibited by BrTu plus Tu, but this effect was reversible and virus growth resumed when the drugs were withdrawn. The complete cure achieved by the combination of analogues appears to be limited to picornaviruses.

**DISCUSSION**

The cure. We describe in this paper the selective inhibition of picornavirus growth by two adenosine analogues, BrTu and Tu. Although no single interpretation emerges uniquely from the available data, we suggest that the antiviral selectivity of the nucleoside mixture is best explained as follows.

In the context of the present experiments, three aspects of BrTu action are important. Firstly, BrTu, an adenosine kinase inhibitor (12, 21), limited the amount of Tu that entered the cellular nucleotide pools in both infected and control cells; indeed, the resulting intracellular Tu nucleotide concentration was too low to inhibit uninfected cell growth. Secondly, since BrTu inhibited the synthesis of high-molecular-weight cellular RNA (11), it protected the cell against damage secondary to Tu incorporation. This facet of twofold protection by BrTu probably accounts for the excellent recovery of uninfected cells from the combined treatment with BrTu and Tu. However, in contrast to its effect on host cell transcription, BrTu did not inhibit nucleotide polymerization by the picornavirus RNA-synthesizing enzyme (12); the latter therefore remained free to incorporate Tu, yielding functionally defective polynucleotides that aborted the virus growth cycle. The most important component in the selectivity of the nucleoside mixture was therefore the ability of BrTu to protect uninfected cells against Tu toxicity.

Several additional factors probably sensitized the susceptibility of the viral replication system to the nucleoside treatment. One of these may have been the location of the viral polymerase (20, 34) in a cellular compartment—the cytoplasm—that includes the nucleoside and nucleotide kinases (12, 21, 22) responsible for converting Tu to the corresponding triphosphate. Another was an as yet unexplained enhancement of Tu metabolism coupled to mengovirus infection that might account for the incorporation of Tu into virus-specific polynucleotides. This change might be due to enhancement of

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**FIG. 6.** Mengovirus-infected cells do not grow after being cured by BrTu and Tu, but uninfected cells resume growth after the same treatment. L-cell monolayers (2 × 10⁵/60-mm-diameter petri dishes) were infected with mengovirus (10 PFU/cell, 60 min, 37°C) in the presence of BrTu plus Tu at 10 and 20 μg/ml, respectively. After adsorption of virus, the cultures were washed and fresh medium containing BrTu and Tu was restored. The drugs were removed from cultures after 2, 4, or 8 h of further incubation. Cells were counted in two defined areas of two plates at each of the indicated time points.
membrane nucleoside transport (14, 19, 35, 36, 38, 42), of phosphorylation (12, 23, 26), or of polymerization of Tu nucleotides in general or to a reduction in the inhibitory action of BrTu and/or its nucleotide derivatives in infected cells, whether at the level of adenosine kinase or other nucleotide-metabolizing enzymes (43). None of these potential mechanisms can be evaluated on the basis of existing data.

Properties of cured cells. Another observation of interest concerns the loss of cell division and the inhibition of RNA synthesis in cured cells. The virtually complete and presumably irreversible block in rRNA synthesis, confirming previous descriptions of mengovirus-infected L cells (3, 7, 18, 25), could be expected to interrupt the normal cell cycle and hence cell division; transcription factors required by RNA polymerase I

TABLE 4. Growth of various viruses in L cells cured of mengovirus infection by the combination of BrTu and Tu

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of PFU/cell in:</th>
<th>Nucleoside-treated, mengovirus mock-infected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control cells (0 h)</td>
<td>Cells cured of mengovirus (24 h)</td>
</tr>
<tr>
<td>Mengovirus</td>
<td>0.175</td>
<td>0.25</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>0.43</td>
<td>0.10</td>
</tr>
<tr>
<td>VSV</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>Reovirus</td>
<td>3.5</td>
<td>1.950</td>
</tr>
</tbody>
</table>

* L-2 cells (4 × 10^6 cells per 60-mm-diameter petri dish) were pretreated for 30 min with BrTu (10 μg/ml) and Tu (20 μg/ml), except for a pair of control cultures, and then all of the plates were infected with mengovirus at an MOI of 10. After virus adsorption for 1 h at 37°C, unadsorbed virus was removed as described in Table 1, footnote a, and fresh medium containing drugs at the concentrations described above was added. Following incubation for 6 h, cells were washed free of the drugs and incubated for a further 90 min in drug-free medium, after which individual pairs of plates of mengovirus-cured cells were superinfected with the indicated virus at an MOI of 10; nucleoside-treated but mengovirus mock-infected cells were also infected in parallel. Following virus adsorption and removal of unadsorbed virus, plates were incubated in drug-free medium for 24 h and then titers were determined as described in Table 1, footnote a.

TABLE 5. Growth of poliovirus in HeLa cells cured of poliovirus or mengovirus infection

<table>
<thead>
<tr>
<th>Virus used for infection</th>
<th>Incubation time (h)</th>
<th>No. of PFU/cell in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells cured of mengovirus</td>
</tr>
<tr>
<td>Mengovirus</td>
<td>24</td>
<td>0.26</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>24</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* HeLa cell monolayers (2 × 10^6 cells per 60-mm-diameter petri dish) were pretreated with BrTu (10 μg/ml) and Tu (20 μg/ml) for 30 min before being infected with either mengovirus or poliovirus at an MOI of 10. At the end of the period of adsorption (1 h at 37°C), the cells were thoroughly washed with medium and incubated for 4 h in the presence of the drugs; two sets of cultures were mock infected but otherwise treated identically. All of the plates were then washed free of the drugs and incubated in fresh drug-free medium for 45 min; the poliovirus-cured cells were then superinfected with mengovirus and vice versa as shown. After the usual period of virus adsorption, all of the plates were washed and incubated in drug-free medium for the indicated times, frozen, and thawed and the virus titer was determined as described in Table 1, footnote a (mengovirus in L cells and poliovirus in HeLa cells). The values in parentheses are the virus yields for accompanying nucleoside-untreated, mengovirus-infected cultures; sufficient intracellular Tu nucleotides were accumulated during the drug treatment to inhibit subsequent virus multiplication significantly under these conditions.
are inactivated during infection by poliovirus (15, 39). Based on the available evidence, it is reasonable to assume that this interruption of RNA synthesis is brought about by the expression of some early viral function—probably a virus-specific protein(s) (16).

The final and most obscure phenomenon in the present work is the surprising resistance of cured cells to superinfection with the same or another picornavirus. There is no basis for preferring any of the numerous potential mechanisms that could account for this picornavirus-specific exclusion or interference. Interference might have resulted from a variety of effects, including interferon production, destruction or blockage of viral receptors located at the host cell surface, or saturation of intracellular sites essential for virus synthesis. Several lines of evidence appear to exclude interferon action as the mechanism responsible for this process. These include the following findings. (i) Resistance develops in the presence of actinomycin, which blocks interferon production, and in addition, the effect occurs more rapidly than would be expected for interferon (28, 44). (ii) Most importantly, the observed resistance is virus specific, whereas interferon would be expected also to block the growth of vaccinia virus and VSV (41).

It is possible that the surfaces of the virus carrier (cured) cells were specifically altered (18) and became refractory to attachment of and/or penetration by closely related viruses. We have, however, ruled out receptor involvement in this exclusion phenomenon by showing that cured cells were not susceptible to superinfection (transfection) with the same or another infectious picornavirus RNA (data not shown). Thus, it can tentatively be concluded that the restricted infection (interference) may be due to consumption or sequestration by the original infecting virus of some limiting cellular element(s) normally required for picornavirus multiplication. Possible candidates are host factors involved in the synthesis of viral RNA (20, 27, 33). It is noteworthy that, whatever the nature of the cellular component, it appears to be irrelevant for viruses other than picornaviruses.

Our findings are encouraging for the ultimate development of specific antiviral chemotherapy. The system we have studied is only a model, and a restricted one at that, and toxicity rules out any immediate practical application of the particular nucleosides we have used. Moreover, although the hypothetical considerations outlined above are attractive, it is probably unsafe to assume that we can account for the antiviral selectivity of the nucleoside treatment entirely in terms of plausible, known mechanisms. In spite of these reservations, the results show that it is possible to exploit observed differences between viral and cellular functions, and between infected and uninfected cells, for the rational design of chemotherapeutic programs that are selectively toxic to the parasite.

### Table 6. Effect of BrTu plus Tu on the growth of different viruses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VSV*</th>
<th>NDV*</th>
<th>Sindbis virus*</th>
<th>Vaccinia virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 h)</td>
<td>0.15</td>
<td>0.04</td>
<td>0.06</td>
<td>1.25</td>
</tr>
<tr>
<td>Control (24 h)</td>
<td>376</td>
<td>188</td>
<td>1,094</td>
<td>62.5</td>
</tr>
<tr>
<td>BrTu (10 μg/ml) (24 h)</td>
<td>400</td>
<td>75</td>
<td>500</td>
<td>1.7</td>
</tr>
<tr>
<td>BrTu (10 μg/ml) + Tu (20 μg/ml) (24 h)</td>
<td>155</td>
<td>12</td>
<td>62.5</td>
<td>0.86</td>
</tr>
<tr>
<td>BrTu (10 μg/ml) + Tu (20 μg/ml) (wash at 8 h)</td>
<td>225</td>
<td>162.5</td>
<td>563</td>
<td>24.4</td>
</tr>
</tbody>
</table>

* Grown in L-2 cells (4 × 10^3 cells per 60-mm-diameter plate).

* Grown in chicken embryo fibroblasts (4 × 10^3 cells per 60-mm-diameter plate).

### REFERENCES


