INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS REPLICATION, CYTOPATHOLOGY, AND PLAQUE FORMATION IN THE PRESENCE AND ABSENCE OF NUCLEIC ACID ANALOGUES

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ABSTRACT

STEVENS, JACK G. (University of Washington, Seattle), AND NEAL B. GROMAN. Infectious bovine rhinotracheitis virus replication, cytopathology, and plaque formation in the presence and absence of nucleic acid analogues. *J. Bacteriol.* **87**:446-453. 1964.—Cytopathology induced by infectious bovine rhinotracheitis (IBR) virus was correlated with the one-step growth cycle. Nuclear alterations, including the development of inclusion bodies, preceded the appearance of virus. It was found that similar effects occurred in the presence of 5-bromodeoxyuridine (BUDR) and 5-fluorodeoxyuridine (FUDR), compounds which depress the yield of "standard" virus from a range of 116 to 500 to less than 0.5 plaque-forming units per cell. As with known members of the herpesvirus group, IBR virus plaques developed and enlarged indefinitely in the presence of specific antibody. An analysis of the mechanism operative in this process was undertaken. The evidence suggested that neither viral nor subviral particles capable of replicating "standard" virus passed between cells during the first 8 hr of infection. This is the time preceding the release of extracellular virus from initially infected cells. With BUDR and FUDR, it was shown that plaques also developed in this system in the virtual absence of production of "standard" infectious virus. However, a class of analogue-dependent virus was found which may have been at least partly responsible for plaque formation in the analogue-treated system. The relative contributions of subviral particles or of a self-sustaining molecular disorganization to the process have not been completely assessed as yet.

We recently described the development of quantitative techniques for the study of infectious bovine rhinotracheitis (IBR) virus,

as well as some of its basic properties (Stevens and Groman, 1963*b*). In the present report, additional characteristics of this virus-host cell system are described. These include a description of cytopathology, and, more particularly, a study of the mechanism of plaque formation in the presence of specific antibody—a property exhibited by at least some members of the herpesvirus group (Black and Melnick, 1955; Wheeler and Canby, 1959; Hoggan and Roizman, 1959). The use of certain nucleic acid analogues in this work led to the discovery of an analogue-dependent virus. A preliminary report of this latter finding was published (Stevens and Groman, 1963*a*).

MATERIALS AND METHODS

Media, cells, and virus. Media, cells, virus, and quantitative techniques were previously described in detail (Stevens and Groman, 1963*b*). In brief, a continuously cultured line of bovine kidney cells (Madin and Darby, 1958) was grown in a modified Earle's salt solution containing lactalbumin hydrolysate (0.5%) and lamb serum (5%). Complete medium will hereafter be designated LA-S. Plaques were developed in a modified H-M medium (Stevens and Groman, 1963*b*) containing 0.6% agar, and were stained with crystal violet (Holland and McLaren, 1959) after 2 days of incubation at 37 C. The virus was a cloned strain used after 20 to 24 passages in tissue-cultured bovine kidney cells.

Cytological techniques. All cytological examinations were made with cells propagated on no. 1 cover slips (7 by 22 mm). Two sterile slips were "glued" with hot 1.8% Noble agar (Difco) to one side of French square bottles (2 oz). Cells were planted as described previously, and grew on both the cover slips and the exposed wall of the bottle. Cover-slip cultures were prepared for microscopy with Bouin's fixation and standard

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H & E staining, a procedure previously described as most efficient for demonstration of IBR inclusion bodies (Stevens and Chow, 1959). Carnoy's fixation and acridine orange staining, as described by Mayor and Diwan (1961), were also used. Here, cover slips were pretreated as described by these authors, placed in 0.01% acridine orange for 7.5 min, mounted in buffer, sealed with melted Vaspar, and examined microscopically with light at a wavelength of 550 m μ .

Antisera. Hyperimmune rabbit serum was obtained from two New Zealand white rabbits which were prebled and inoculated intravenously at biweekly intervals with a total of 8×10^7 plaque-forming units (PFU) of virus over a period of 4 weeks. The rabbits were bled, and the serum was titered after 2 additional weeks. This serum possessed a *K* value (Adams, 1959) of 7.2 when incubated at 37 C with the virus.

Bovine antiserum at a 1:500 dilution inactivated 150 of 200 PFU of virus when incubated at 37 C for 1 hr.

All antisera were inactivated at 56 C for 30 min before use, and were stored at -20C.

Removal of residual, uneclipsed surface virus after adsorption. In experiments involving nucleic acid analogues, uneclipsed surface virus remaining after adsorption had to be effectively removed. Gresser and Enders (1961) reported that trypsin destroyed herpes simplex virus. In the present system, the level of cell-associated IBR virus was significantly reduced by treatment with trypsin. Therefore, trypsin was used to reduce extracellular cell-associated virus in all inhibitor experiments. When trypsin was used, cultures were rinsed once with LA-S (5 ml), rinsed with 1 ml of trypsin solution (Difco, 1:250, 0.05% in phosphate-buffered saline), incubated at 37 C with an additional 2 ml of trypsin for 2 min, rinsed again with 5 ml of LA-S, and overlaid with appropriate media. This treatment did not affect the yield of virus in control cultures.

Determination of extracellular, intracellular, and total virus. It became important in experiments employing analogues to use the most efficient method of releasing virus from cell debris. Sonic treatment (Raytheon, 10 kc, 2- to 4-ml samples in 12-ml polypropylene rubber-stoppered tubes immersed in 25 ml of water inside the cup) for 0.5 to 4 min was compared with freezing and thawing (-70 $\rightarrow$ 37 C) from zero to seven times for ability to release virus. Sonic treatment

for 1 min was as good as, or better than, the other methods for determining maximal amounts of virus (Stevens and Groman, *unpublished data*). Except where noted otherwise, this method was used to release virus in all experiments.

Analogue treatment of monolayers. Unless stated otherwise, in all experiments where 5-bromodeoxyuridine (BUDR) was used, monolayers were pretreated with the analogue for 36 or 50 hr before virus adsorption. After adsorption, the analogue was usually readded, either alone or in combination with thymidine or 5-fluorodeoxyuridine (FUDR).

RESULTS

Inhibition of IBR virus by BUDR. BUDR belongs to a group of halogenated pyrimidine deoxyribonucleosides which inhibit deoxyribonucleic acid (DNA)-containing animal viruses, including members of the herpesvirus group (*see* Salzman, 1960; Herrmann, 1961; Kaufman and Maloney, 1963). If this analogue interfered with the IBR virus, it could serve as a tool to study the relationship between cytopathology, plaque formation, and infective virus production.

First, the effects of BUDR on cell multiplication were studied. Cell numbers in both BUDR-treated (30 μ g/ml) and control monolayers increased by more than 3.5 times in 50 hr, and, at the 50th hr, cell numbers in treated cultures were about 80% those of controls. This indicated that the Madin-Darby bovine kidney (MDBK) cell line divided at least two times at a relatively normal rate in the presence of BUDR. Because cell division was not significantly inhibited by BUDR, cells were pretreated with the analogue to increase the intracellular concentration of BUDR before viral adsorption.

The effect of BUDR on infective virus production was determined. The results of two experiments (Table 1) show that virus yield in the presence of BUDR was depressed greater than 500-fold. This depression was, to a great extent, reversed by thymidine at 125 μ g/ml. The fact that pretreated cells produced considerable amounts of virus when incubated in inhibitor-free medium indicated that the virus-producing capacity of the cells was not irreversibly altered by pretreatment with BUDR. It appears from the second experiment that pretreatment of monolayers was not essential for BUDR inhibition of virus production, a result which was

TABLE 1. *Effects of 5-bromodeoxyuridine (BUDR) on IBR virus production in monolayers infected at a high multiplicity of infection**

Preadsorption treatment	Postadsorption treatment	Virus produced per cell	
		Expt 1	Expt 2
LA-S	LA-S	110	240
LA-S + BUDR	LA-S + BUDR	0.2	0.47
LA-S + BUDR	LA-S + BUDR + thymidine†	60	163
LA-S + BUDR	LA-S	65	168
LA-S	LA-S + BUDR	—	0.58

* BUDR-pretreated (30 $\mu\text{g/ml}$, 50 hr before infection) and control monolayers were infected at a multiplicity sufficient to insure infection of all cells. These monolayers were washed three times with LA-S, and subjected to various treatments after infection. Virus titers were determined 24 hr after infection after 4 cycles of rapid freezing and thawing ($-70^\circ\text{C} \rightarrow 37^\circ\text{C}$).

† Thymidine = 125 $\mu\text{g/ml}$.

FIG. 1. *Relationship between growth of IBR virus and cytopathology. At least 1,000 random cells (on slides from duplicate cultures) were observed and classified for each time period noted.*

confirmed in subsequent experiments. Finally, it was found that BUDR at 120 $\mu\text{g/ml}$ did not affect free virus titers over a 24-hr period at 37°C .

Correlation of cytopathology with the one-step growth cycle in untreated and analogue-treated cultures. Control and analogue-pretreated monolayers were infected at a high multiplicity, treated with trypsin, and incubated at 37°C . Periodically, duplicate bottles (two with cover slips and two without) from both groups were removed from the incubator. Those bottles without cover slips were frozen at -70°C , and were subsequently assayed for total virus, whereas cover-slip cultures were fixed in Bouin's fixation and stained with H & E staining. Cells on cover slips were counted and placed into one of three categories: "normal," cells with inclusions (single, multiple, and "haloed"), and rounded cells with pyknotic nuclei. The results of a representative experiment with untreated cells (Fig. 1) show that, as the infective cycle progressed, there was a general trend from a population of "normal" cells to one with cells exhibiting pyknotic nuclei. Beyond 8 hr, the relative numbers of round cells increased, but constriction of monolayers, aggregation of nuclei, and massive detachment of cells led to inaccurate counts. Eventually, all cells floated free in the medium. Total virus titer did not rise until inclusion bodies had formed in over half of the cells, and until some round pyknotic cells were present. It appears, therefore, that inclusion formation precedes virus maturation, and that maturation correlates with the appearance of rounded cells with pyknotic nuclei. Low counts of round cells at all stages of the cycle probably indicate the rapidity with which these cells are released from the glass after their formation. It should also be noted that over 95% of all cells in infected cultures exhibited inclusions by 8 hr, indicating that cells were uniformly susceptible to infection. On the average, 450 PFU of virus were produced per cell at 20 hr, the time at which maximal virus levels were reached in this experiment.

Cytopathology and virus production were also followed in analogue-treated cultures in which both BUDR and FUDR were used. FUDR was used because it inhibits thymidylate synthesis (Bosch, Harbers, and Heidelberger, 1958; Cohen et al., 1958), and may have potentiated the BUDR effect in this system by forcing cells to use BUDR in place of any thymidine which was available in cells not treated with FUDR. In a typical experiment, monolayers were pretreated for 36 hr with 100 $\mu\text{g/ml}$ of BUDR, and all cells were infected. After trypsin treatment, 6 ml of

LA-S with 100 $\mu\text{g}/\text{ml}$ of BUDR and 10 $\mu\text{g}/\text{ml}$ of FUDR were added. Samples for titration and staining were taken at the same time as for untreated cultures. Inhibition of virus synthesis by these methods was not complete, for a small rise in titer occurred between 4 and 20 hr (from 2.5×10^4 to 1.1×10^5 PFU). As in uninhibited cultures, this rise was most dramatic between 4 and 8 hr. Despite the fact that total virus remained considerably below the total number of cells in the monolayer (0.25 PFU per cell at 20 hr), more than 90% of the cells exhibited inclusions by 8 hr. Thus, though nearly all cells were infected (as evidenced by the presence of inclusion bodies), on the average at least 70% were not virus producers. In general, classification of cells after infection revealed the same pattern of development as was seen in untreated cultures, except that inclusion bodies were usually less dense and smaller and the overall sequence of degeneration occurred more slowly. As an example, only 83% of the inhibitor-treated cells exhibited inclusions at 6 hr, compared with 92% in untreated cultures.

Representative fields of noninfected cells and inclusion bodies in cells from infected controls are shown in Fig. 2. It should be emphasized that percentages of cells in different stages of infection varied slightly from experiment to experiment, but the trends were constant, and formation of inclusion bodies always preceded virus production. Inclusion bodies were also seen in nearly all cells that had been inhibited with BUDR only (30 or 100 $\mu\text{g}/\text{ml}$), a situation in which less than 0.5 PFU of virus were produced per cell.

When infected cultures were stained with acridine orange, the untreated and BUDR-FUDR treated cells revealed a similar pattern of cytopathogenicity. Chromatinic materials were margined, nucleoli were fragmented and margined, and a very few greenish-yellow (DNA?) inclusions were visible in only a few cells. In most infected cells, a dark unstained area was observed in the nuclear area at the time when inclusions were seen in cells stained by the Bouin-H & E process. This result was not unexpected, because Carnoy's is known to be a relatively poor fixative for IBR inclusion bodies (Stevens and Chow, 1959), but indicated that material in the inclusion area was not nucleic acid, or that nucleic acid, if present, was either not available for staining or was present in

FIG. 2. Inclusion body formation by IBR virus in inhibited and uninhibited MDBK-cell cultures. (A) Uninhibited, 8 hr after virus infection. (B) Inhibited (BUDR and FUDR), 8 hr after virus infection. All specimens were fixed in Bouin's fluid and stained with H & E. Magnification, 560 $\times$. I = intranuclear inclusion bodies.

relatively small quantities. Again, the degree of involvement in individual nuclei appeared to be less dramatic in cultures treated with BUDR and FUDR, and the time of appearance of various effects was delayed.

In general then, IBR virus production begins about 4 hr after infection. At this time, 50% or more of the cells contain intranuclear inclusions, some cells are shrinking, and their nuclei are becoming pyknotic. The same general pattern is seen in cells treated with BUDR and FUDR. However, instead of a yield of at least 200 PFU of virus per infected cells, the yield in inhibitor-treated cultures was less than 0.5 PFU per cell. Therefore, cytopathic effects are largely divorced from the production of infective virus under conditions of analogue treatment.

Plaque formation by IBR virus in the presence of antiserum. As noted earlier, herpesviruses produce plaques that enlarge in the presence of excess antiserum. Experiments were performed

TABLE 2. *Plaque development by IBR virus in the presence of antiserum**

Postadsorption medium	Avg no. of plaques	
	Expt 1	Expt 2
H-M + agar	50	64
LA-S + IBR antiserum	50	63

* Virus was adsorbed in the usual manner, and monolayers were overlaid with either H-M agar medium or LA-S medium containing 10% anti-IBR rabbit antiserum. Plaques developed under H-M were stained and counted after 2 days, whereas those produced in the presence of antiserum were processed after 2 or 3 days of incubation, when they were large enough to count accurately.

TABLE 3. *Plaque development by IBR virus in the presence of BUDR, FUDR, and antiserum**

Titration medium	Avg no. of plaques		
	Expt 1	Expt 2	Expt 3
LA-S + BUDR, FUDR, antiserum	75	122	84
LA-S + antiserum	74	108	88
H-M + agar	58	112	83

* A countable number of PFU were permitted to adsorb to pretreated (100 µg/ml of BUDR, 36 hr) and nonpretreated monolayers, which were then overlaid with the appropriate medium. The test group (pretreated with BUDR) received BUDR (100 µg/ml), FUDR (10 µg/ml), and bovine antiserum (1:100); nonpretreated monolayers (controls) received H-M with agar or 1:100 bovine antiserum in LA-S. Plaque counts were determined after 2 days in control bottles and after 3 days in cultures containing analogues.

to see if IBR virus could also form plaques in an immune system. The results of two experiments (Table 2) show that the efficiency of plating in antiserum-treated cultures is equivalent to that in agar-overlay controls. It should also be mentioned that supernatant fluids from cultures exposed to antiserum for 72 hr during plaque development contained no infectious virus. This shows that the inhibition of extracellular virus by antiserum was complete. In these and the inhibitor experiments to be described later, secondary plaques (probably initiated by infected cells which became free and then reattached) began to develop in the antibody-treated mono-

layers by the time the plaques were stained. However, they were small and could easily be differentiated from primary plaques.

Attempts to detect passage of infectivity before extracellular release of virus. An effort was made to detect the spread of infectivity prior to the release of extracellular virus. In these experiments, replicate monolayers were infected at low multiplicity and, at various times prior to the onset of virus release (up to 8.5 hr after virus adsorption), cells were dispersed. One sample of dispersed cells was assayed for infective centers. A second sample was incubated in liquid medium for 24 hr, long enough to permit a full reproductive cycle, and was then assayed for total virus. It was reasoned that if cell to cell spread occurred, then the infective center count and the total virus yield would at some point exceed that expected on the basis of input virus alone. A number of such experiments were performed. In general, the infective-center counts actually showed a decrease over the initial levels, and the total virus yields also declined and then rose, but never exceeded the yield obtained from the initial sample. Preferential lysis or destruction of infected cells due to handling was ruled out by making comparative counts on the infected and uninfected cell suspensions. The data suggest that spread of infectivity does not occur within the 8.5-hr period studied, but does not exclude cell to cell spread at a later time. It also does not exclude the possibility that cells treated in this manner are damaged to the extent that virus reproduction cannot take place.

Analysis of plaque development in the presence of BUDR, FUDR, and antiserum. As noted earlier, BUDR (100 µg/ml) and FUDR (10 µg/ml) reduce the yield of infectious virus to less than 0.5 PFU per cell in cultures where all cells are infected. This suggested a method of determining the contribution of mature infectious virus to the process of plaque development in immune systems. If plaques developed in cultures containing antibody plus inhibitors which limited virus reproduction to less than 0.5 PFU per infected cell, and if plaque counts approximated those of controls without inhibitors, then a process other than passage of mature infectious virus could be considered to be operative in the system.

Results of three experiments testing the effects of antibody and inhibitors on plaque production are given in Table 3. These data show that

plaque efficiency was the same in control and test groups. The experiments suggested that IBR virus plaques developed in MDBK cell monolayers by a process not involving the production of "standard" infectious virus. As a check on inhibitor effectiveness, additional monolayers were infected at high multiplicity, residual virus was removed with 0.05% trypsin, and the BUDR-FUDR combination was added. Total virus yield was determined on sonically treated or frozen and thawed material after 24 hr at 37 C. These infected cells, on the average, produced less than 0.2 infective viruses per cell.

The effectiveness of inhibitors under conditions of low multiplicity and over the 72-hr period of infection, conditions in effect in the present experiments, was also tested. The same amount of virus was produced in both groups, proving that original concentrations of inhibitors were effective during the 72-hr plaque-forming period (Table 4).

Inhibitor-dependent virus. The results presented in Table 4 reveal the important fact that the viral genome was replicating over the 72-hr period in the presence of inhibitors. Whereas the output-input ratio is the expected 0.2 at 24 hr, it rose to about 3 at 48 hr, and to 10 at 72 hr. Such an increase could only occur if a drug-mediated modification of cells or virus took place, or if an inhibitor-resistant virus was present in the inoculum or was produced during the early phase of limited reproduction. As was reported in a preliminary publication (Stevens and Groman, 1963a), an inhibitor-dependent virus was produced in this system. This virus appears after one replication cycle in inhibitor-treated cells and plates 37 to 87 times more efficiently in the presence of inhibitors than in their absence. It is also clear that this virus may have some bearing on development of plaques in the inhibited system.

DISCUSSION

As stated earlier, selective inhibition of DNA viruses by FUDR or BUDR was reported many times. The effect of BUDR on IBR virus replication in MDBK cells permits a number of assessments to be made regarding this virus-host cell system. Thus, inhibition of IBR virus by these compounds strongly suggests that this virus is a DNA virus, as are prototype herpesviruses (Ben-Porat and Kaplan, 1962). Reversal of the BUDR effect by thymidine attests to the specificity of the reaction. The occurrence of

TABLE 4. *Effect of renewal and nonrenewal of inhibitors on IBR virus production**

Time	PFU per monolayer (LA-S + BUDR-FUDR)	
	Inhibitors renewed	Inhibitors not renewed
hr		
0†	0	0
24	33	33
48	480	600
72	1,750	1,840

* Pretreated monolayers were infected with 175 PFU of virus, washed with trypsin, and overlaid with LA-S containing BUDR and FUDR. To one group, 100 μ g of BUDR and 10 μ g of FUDR were added 24 and 48 hr after infection, whereas the other group received no additional inhibitors. Duplicate bottles were taken from each group at 24-hr intervals, frozen, and later sonically treated and titrated.

† After adsorption.

cytopathology and cytopathology with inclusion body formation in the presence of compounds inhibiting the production of mature virus is not a new observation (*see* discussions by Loh, 1960; Reissig and Kaplan, 1962). However, to our knowledge, development of intranuclear inclusion bodies was not reported in virus-infected cells inhibited with BUDR. This compound, which is incorporated into DNA, has an action different from that of other inhibitors (FUDR, amethopterin, and parafluorophenylalanine) which are also able to divorce cytopathic effect from virus production in cell culture systems.

The relationship of virus to intranuclear inclusion bodies was illuminated to some extent by the use of inhibitors, as well as by other observations. Inclusion bodies were formed in all cells of inhibitor-treated cultures where less than 15 PFU of virus were produced per cell. This suggests that such inclusions are principally fabricated from substances other than "standard" mature virus particles. The finding that inclusions formed before infectious virus could be detected also supports such a view. Furthermore, in most cases inclusion bodies, or material in inclusion areas, could not be identified in cells processed by the acridine orange technique, a process which identifies both types of nucleic acid. In such cells, nucleic acids were unavailable for staining or not present in the inclusion areas. However, abundant DNA fluorescence near the inner surface of the nuclear membrane suggested

that active DNA synthesis may have been taking place in this area of margined chromatin rather than in inclusion areas. Feulgen-staining of IBR inclusion bodies has given equivocal results. They have been reported as Feulgen negative (Orsi and Cabasso, 1958) and positive, apparently because of different fixatives used (Liess, Knocke, and Schimmelpfening, 1960). Ultrathin sections of uninhibited IBR virus-infected cells examined by electron microscopy showed a relatively low density of virus-like particles in inclusion areas, suggesting inclusions are not largely composed of virus particles (Armstrong, Pereira, and Andrewes, 1961). It must be remembered, however, that sections shown may not have been cut in areas where virus density was greatest. In general, these findings are consistent with the interpretation that IBR inclusion bodies mainly contain virus or cellular protein material that has become coagulated and rendered visible by fixation procedures, or both. [Both Zenker's and Bouin's solutions contain such coagulating compounds (Baker, 1960).] Such an interpretation is also supported by the observation that inclusion bodies develop in situations where "coreless" pseudorabies virus is produced (Reissig and Kaplan, 1962).

The finding that plaques develop in an immune environment and in such an environment where virus production is significantly inhibited by BUdR and FUDR extends the observations of others who studied herpesvirus plaque development in immune systems (Black and Melnick, 1955; Wheeler and Canby, 1959; Hoggan and Roizman, 1959). At least three processes, acting alone or in combination, could be operative during such plaque formation. First, a considerable amount of normal but unassayable infective virus may be produced. This virus could pass from cell to cell, giving plaques. Although considerable effort was spent in developing the most sensitive assay method, such a process cannot be ruled out at the present time. A second explanation involves postulating that subviral particles are passing between contiguous cells. This hypothesis has not been borne out by the present experiments, although it has not been disproved either. If such particles exist, they probably pass after the development of infectious virus, because experiments indicated that such passage does not occur before the release of virus from infected cells. A third process

which could be operating is a toxic process. This may involve either a "classic" toxin which could spread to adjacent cells and kill them, or a molecular disorganization which could be self-sustaining from cell to cell. For example, disorganization of the surface membrane of the initially infected cell may induce a similar change in contiguous cells. Such membrane disorganization, once started, could conceivably spread by extension, resulting in a plaque. Membrane alterations induced by herpes simplex virus were studied in detail by Roizman (1962). Such alterations, which could involve toxic-like processes or the presence of virus, or both, result in the formation of polykaryocytes and plaques. Association of a "classic" toxin with IBR virus is questionable, because certain experiments (Stevens and Groman, *unpublished data*) failed to reveal such a toxin. A self-sustaining molecular disorganization cannot be ruled out at this time.

It was hoped that the use of nucleic acid inhibitors would lead to a clear-cut decision regarding the role of mature virus in plaque production in an antibody-containing system. The discovery of inhibitor-dependent virus leaves this issue in doubt, because dependent virus may be responsible for plaque formation in the inhibited system. This virus, like normal virus, may pass in some form directly from cell to cell in infected systems.

From the foregoing considerations, then, it is obvious that several processes may be operative during plaque formation in the analogue-inhibited or antibody-containing cultures, or both. With nucleic acid inhibitors, the contribution of normal infective virus to the process was minimized or possibly eliminated. The production of a classical extracellular toxin by the initially infected cell is questionable, but has not been completely ruled out. Analogue-dependent virus in either complete or incomplete form may contribute to plaque production in the inhibited system. Finally, a toxic process involving progressive rearrangement at the molecular level or the passage of a subviral particle, or both, are alternatives still to be seriously considered.

Variations of the present technique of employing inhibitors may be useful for further investigations of cell to cell transfer of cytopathology. For example, other nucleic acid inhibitors (as FUDR alone or amethopterin) or protein inhibitors (as parafluorophenylalanine or puromycin)

cin) could be used profitably in studying this problem.

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