Complete Cure of DNA Virus Infection in Cultured Cells by Drug Combinations.* (29414)

EIICHI FURUSAWA, WINDSOR CUTTING, PATRICIA BUCKLEY AND SHINOBU FURUSAWA
Pacific Biomedical Research Center, University of Hawaii, Hawaii

In a previous paper(1) it was shown that 5-methyl-tryptophan (5-me-TP) and noformicin (NOF), an antibiotic, N-(2-amidinoethyl) - 5- imino - 2 - pyrrolidine - carboxamide (15), out of 60 selected compounds, showed remarkable activities against vaccinia propagation in HeLa cells; NOF was also active against adeno type 12 virus. In this paper, we show that the combinations of 5-me-TP and 5-bromo-deoxyuridine (5-BUDR), or NOF and 5-BUDR, can completely suppress vaccinia growth and eradicate the virus from infected cultures. Normal cell population is restored after removing the drug combinations from the culture medium; this does not occur after single drug administration. Against adeno type 12 virus complete eradication of the infection was doubtful even with the drug mixtures.

Materials and methods. 1. *Virus-host systems.* Vaccinia, IHD strain, and adeno virus type 12 (Huie) were used; each was passed in HeLa cells about 10 times before use. Virus titers were determined with 2 tubes for each 10-fold dilution, and the highest dilution which showed 4+ cytopathic effect (CPE) in both tubes was taken as the 100% tissue culture infectious dose ($TCID_{100}$ or TCD). HeLa cells were grown at 36°C in stationary culture in medium 199 containing 2% inactivated calf serum. Virus inoculation and drug administration were usually done at the same time in HeLa cells which were freshly trypsinized and suspended at a concentration of 5×10^4 cells per ml in 1 ml of culture medium per tube. The monolayers of cells were formed 5 days later. CPE of the viruses as judged by microscopic examination was recorded as 0, 1+, 2+, 3+, and 4+ degrees.

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2. *Antiviral testing of compounds.* Test compounds were kept in Hanks' solution at 4°C; 0.1 ml was added to each culture tube. Effects of the compounds on cells were evaluated in non-infected cultures in the following way: A concentration of compound which permitted formation of the cell sheet, but which caused a gradual loss of cell numbers during the 10- to 12-day exposure to the compound, was taken as the minimum toxic dose (MTD). At one-half this level, designated as the maximum non-toxic dose (MNTD), the monolayer persisted without increase or decrease over the cell numbers in the inoculum throughout the incubation time. This was ascertained by counts performed on replicate tubes at intervals up to 12 days. In assigning anti-viral activity of each compound, a concentration was chosen which was one-fourth the MTD, or less; hence, one-half the MNTD, or less. For example, the MTD for 5-BUDR was 1.6 μg/ml; for 5-me-TP it was 800 μg/ml; and for NOF it was 10 .g/ml. At twice the level of MTD, no confluent cell sheets were formed and cells degenerated and fell off the glass. Compared with monosheets of cells which had been cultured for several days from seeding, freshly trypsinized cells were more sensitive to the compounds. Therefore, the minimum toxic doses were lower than when determined against established monosheets of cells.

Results. a. *Antiviral effects of NOF and 5-BUDR against adeno type 12 propagation before and after appearance of CPE.* Single drug treatment was started at 0, 3 and 6 days after infection. Medium including drug was changed 3, 5, 6, and 7 days after infection; incubation was for 9 days at which time the control cultures showed maximum CPE and virus titer. Table I shows the result of treatment. NOF showed a partial inhibitory effect when it was administered to the infected cells after CPE had already appeared, but 5-BUDR showed the partial effect only when

TABLE I. Antiviral Effects of Noformicin and 5-Bromodeoxyuridine Against Adeno Type 12 Propagation in HeLa Cells Before and After Appearance of CPE.*

Day treatment started after infection	CPE before drug added	5-BUDR .4 μg CPE	5-BUDR .4 μg TCD	5-BUDR .2 CPE	5-BUDR .2 TCD	Noformicin 2.5 μg CPE	Noformicin 2.5 μg TCD	Noformicin 1.2 CPE	Noformicin 1.2 TCD	Noformicin .6 CPE	Noformicin .6 TCD	No drug CPE	No drug TCD
0	0	2+	3	4+	5	0	1	0	1	1+	2	4+	5†
3	0	4+	4	4+	5	1+	2	1+	1	2+	3	4+	5
6	2+	4+	5			2+	3	2+	3	4+	5	4+	5

* Inoculated with 100 $TCID_{100}$ of virus and incubated for 9 days. Medium was changed 3, 5, 6, 7 days after infection.

† 5 indicated a virus titer of 10^5 $TCID_{100}$ per 0.1 ml of medium.

TABLE II. Delayed Activities of 5-Bromodeoxyuridine and Noformicin Against Adeno Type 12 Infection in HeLa Cells During an Extended Incubation Period.*

		Incubation period 9 days CPE	9 days TCD	11 days CPE	11 days TCD	13 days CPE	13 days TCD	15 days CPE	15 days TCD	17 days CPE	17 days TCD
5-BUDR	.4 μg	2+	3	2+	3	3+	3	4+	3		
Noformicin	2.5	0	1	1+	2	2+	2	3+	4	4+	4
	1.2	0	1	1+	2	3+	3	4+	4	4+	4
	.6	1+	2	2+	3	4+	5				
No drug		4+	5								

* Drugs are added just after inoculation of 100 $TCID_{100}$ of adeno type 12 virus. Medium including drugs was changed every 2 days.

it was administered at time of virus inoculation.

b. *Failure of final eradication of adeno type 12 infection with further incubation.* As shown in Table I, definite suppression of virus growth occurred in infected cultures when drug administration was started at time of infection. However, when the cultures were incubated for 8 further days after complete CPE in the control (Table II) neither NOF nor 5-BUDR could suppress virus propagation or CPE, and could not save the cells from final death.

c. *Antiviral effects of 5-me-TP, NOF and 5-BUDR against vaccinia propagation before and after appearance of CPE.* The experiment was performed in the same way as for adeno type 12 (Table I). It was found that 5-me-TP and NOF showed definite partial effects against further CPE in infected cell cultures in which CPE had already appeared, and 5-BUDR showed a doubtful effect (Table III).

d. *Failure of final eradication of vaccinia with further incubation.* The experiment was performed in the same way with adeno type

TABLE III. Antiviral Effects of 5-Bromodeoxyuridine, 5-Methyltryptophan and Noformicin Against Vaccinia Propagation in HeLa Cells Before and After the Appearance of CPE.*

Day treatment started after infection	CPE before treatment	5-BUDR .4 μg CPE	5-BUDR .4 μg TCD	5-BUDR .2 μg CPE	5-BUDR .2 μg TCD	5-me-TP 200 μg CPE	5-me-TP 200 μg TCD	Noformicin 1.2 μg CPE	Noformicin 1.2 μg TCD	No drug CPE	No drug TCD
0	0	0	0	2+	4	0	0	0	0	4+	6
4	1+	3+	5	4+	6	2+	4	3+	4	4+	6
5	2+	3+	5	4+	6	2+	3	2+	3	4+	6
7	3+	4+	5	4+	6	3+	4	3+	4	4+	6

* Cell cultures were inoculated with 100 $TCID_{100}$ of IHD strain and incubated for 9 days. Medium was changed every other day.

TABLE IV. Delayed Activities of 5-Bromodeoxyuridine, 5-Methyltryptophan and Noformicin Against Vaccinia Infection in HeLa Cells During an Extended Incubation Period.*

		Incubation period									
		8 days		10 days		12 days		14 days	16 days	18 days	20 days
		CPE	TCD	CPE	TCD	CPE	TCD	CPE	CPE TCD	CPE TCD	CPE TCD
5-BUDR	.4 μg	0	0	0	0	0	0	0	2+ 2	3+ 3	4+ 3
5-me-TP	200	0	0	0	1	2+	4	3+	3+ 5	4+ 5	
Noformicin	1.2	0	0	0	1	1+	1	1+	2+ 4	3+ 5	4+ 6
No drug		4+	6								

* Drugs were added just after inoculation of 100 $TCID_{50}$ of IHD strain; medium including drugs was changed every 2 days.

12 (Table II). As shown in Table IV, these compounds could not suppress the propagation of CPE and virus reproduction, and could not save the cells from final death when the infected cultures were incubated 12 more days after completion of CPE in the control, even when the treatment was started at time of infection.

e. *Eradication of vaccinia virus from infected cell cultures and recovery of normal cells through treatment with drug combinations.* From the experiments mentioned above it was concluded that administration of single drugs was not sufficient to eradicate virus from infected cultures. Therefore, treatments with drug mixtures were performed. NOF, 5-me-TP, and several compounds tried previously against vaccinia infection(1), indoloquinoxaline, 5-nitrobenzotriazole, vitamin B_{12} and propionin, which may inhibit virus protein synthesis, were tried in combination with 5-BUDR which attacks viral DNA synthesis. Treatment with the drug mixtures was started at the time of vaccinia virus inoculation. Medium including the mixtures was changed every 2 or 3 days during 16 days of cultivation; then the drugs were removed and the cultures were continued for 2 more weeks. Table V shows the results. When mixtures of 5-me-TP and 5-BUDR, each at ¼ MTD, or NOF at ¼ or ⅛ MTD and 5-BUDR at ¼ MTD were tested as described, no CPE was observed 16 days of exposure to these mixtures, nor at any time during the next 29 days of observation. Growth of cells after drug withdrawal necessitated subculture at 33 days, and periodically thereafter. All fluids harvested during the whole time failed to initiate infection in fresh cultures, even when used undiluted for inocula. In contrast, as seen in Table IV, cultures uniformly showed 4+ CPE and moderate to high infectivity by the 20th day in the presence of the same concentration of any single compound tested. The mixtures, tested in non-infected cultures, allowed maintenance of cells in good monosheets with slow production of acid and no gain or loss in cell numbers over the inoculum at the time drugs were removed from the medium. Infected cultures showed a lag in acid production and cell multiplication for a week after drug removal, and then appeared to resume normal rates and could be subcultured as before. Subcultures showed no resistance to reinfection with vaccinia virus.

f. *Dose response in drug combinations curing vaccinia-infected cultures.* Combinations made with various doses of 5-me-TP, NOF

TABLE V. Eradication of Vaccinia from Infected Cultures and Recovery of Normal Cells by Treatment with Drug Combinations.

Agent*		Viral CPE during incubation period with drug					Viral CPE during incubation period without drug										
							Subculture of cells										
		6	8	10	12	14	16	18	21	24	27	30	33	36	39	42	45 days
no drug		2+	4+(6)†														
indoloquinoxaline	20 μg	0	0	1+	3+	4+(5)											
5-nitrobenzotriazole	5 "	0	0	2+	4+(6)												
vitamin B_{12}	25 "	0	0	2+	4+(6)												
propionin (P8B1)	.1 ml	0	0	2+	4+(6)												
5-methyltryptophan	200 μg	0	0	0	2+	4+(6)											
noformicin	2.5 "	0	0	0	1+	3+	4+(6)										
	‡ 1.2 "	0	0	0	2+	3+	4+(6)										
5-bromodeoxyuridine	.4 "	0	0	1+	3+	4+(5)											
5-bromodeoxyuridine plus:	.4 "																
indoloquinoxaline	20 "	0	0	0	0	1+	3+4+(5)										
5-nitrobenzotriazole	5 "	0	0	1+	4+(5)												
vitamin B_{12}	25 "	0	0	1+	3+	4+(5)											
propionin (P8B1)	.1 ml	0	0	1+	1+	2+	4+(5)										
5-methyltryptophan	200 μg	0	0	0	0	0(−)	0	0	0	0	0	0	0	0	0	0	0(−)
noformicin	2.5 "	0	0	0	0	0(−)	0	0	0	0	0	0	0	0	0	0	0(−)
	‡ 1.2 "	0	0	0	0	0(−)	0	0	0	0	0	0	0	0	0	0	0(−)

* Drugs were added just after inoculation of 100 $TCID_{100}$ of IHD strain of vaccinia, medium was changed every 2 or 3 days.
† (6) indicates virus titer of 10^6 $TCID_{100}$ per 0.1 ml in culture medium when the CPE reached 4+ degree. (−) indicates no virus detection in medium.
‡ One-eighth dose of MTD, the others were all one-fourth of MTD.

TABLE VI. Dose Response of Drug Combinations in Cure of Vaccinia-Infected HeLa Cell Cultures.

Combination*		5-bromodeoxyuridine .8 μg	.4	.2	noformicin 2.5 μg	1.2	.6	no drug
5-methyltryptophan	400 μg	R†	R	20‡	22	22	20	19
	200	R	R	20	18	18	16	14
	100	R	20	14	18	18	12	12
	50	22	17	12				10
noformicin	2.5 μg	R	R	20				20
	1.2	R	R	19				18
	.6	R	R	16				14
	.3	R	20	14				12
	.15	20	16	14				9
no drug		19	16	12				7

* Drug combinations were added just after inoculation of 100 $TCID_{100}$ of IHD strain; medium containing the drugs was changed every 2 or 3 days during 2 weeks, then drugs were removed. Cultures were continued for about 30 days until they showed good growth.

† R indicates recovery of cells; that is, prevention of virus-induced CPE with subsequent good cell growth and satisfactory subculture.

‡ Number indicates the day when viral CPE reached 4+ degrees.

and 5-BUDR were administered just after inoculation of 100 $TCID_{100}$ of vaccinia in experiments as described before. The medium containing the combinations was changed every 2 or 3 days during 15 days' cultivation; then the drugs were removed. Table VI shows the effective range of the combinations for eradication of virus. It was found that various combinations of NOF and 5-BUDR showed definite effective ranges, but that combinations of NOF and 5-me-TP were unable to eradicate the virus in spite of long term suppression. Minimum toxic dose (MTD) of the mixtures against HeLa cells was determined 10 days after incubation as described previously. At one-half of MTD, all of the drugs, except NOF, had produced no pathological changes and allowed a good monosheet for long periods of cultivation until the growth of cells required subcultures. NOF showed a definite toxicity (pyknosis and karyorexis) at one-half MTD (5 μg/ml) when the culture was continued over 2 weeks. Therefore, NOF was used at one-fourth (2.5 μg/ml) of MTD for long term cultivation. At one-half (0.8 μg/ml) and one-fourth (0.4 μg) of MTD of 5-BUDR, cultures stayed as good monosheets without cell growth or acid production for about 10 days and then cell growth and acid production started very slowly. At one-half (400 μg/ml) of MTD of 5-me-TP and one-fourth (2.5 μg/ml) of MTD of NOF, cells could slowly propagate with decreased acid production from the beginning of the cultivation. When 5-BUDR was mixed with 5-me-TP or NOF at each maximum non-toxic dose (MNTD, one-half of MTD), cell propagation and acid production were completely suppressed for a month with good monosheet and without increased toxicity. After removing the drug mixtures, cells propagated slowly and then recovered normal growth-rate and acid production within 2 weeks. Infected cultures containing the drug mixtures (5-BUDR with 5-me-TP or NOF) behaved the same as non-infected cultures in the mixtures. With combinations of 5-me-TP and NOF at each MNTD, cell growth was not completely suppressed from the beginning of cultivation. Thus, it appeared that the vaccinia-infected cultures had to be exposed to doses of drug mixtures which could markedly, if not completely, suppress the cell metabolism if eradication of virus were to appear.

g. *Failure of eradication of adeno type 12 virus.* Contrary to the results with vaccinia virus, no combination of 5-BUDR, 5-me-TP or NOF eradicated adeno type 12 virus from infected cells. Even under the apparently complete suppression of cell growth with mixture of 5-BUDR and NOF at MNTD, CPE accompanied by virus production appeared 7 to 10 days after completion of CPE

in the control. 5-me-TP did not increase the effects of 5-BUDR or NOF against adeno type 12 propagation.

Discussion. There are many reports of successful virus chemotherapy in culture cells using the criteria of inhibition of virus growth and CPE at the time when the control cultures had reached maximum virus growth and CPE. However, there are few reports which describe the fate of the infected cultures after a long period of subsequent cultivation with or without drugs. From the standpoint of virus chemotherapy, it would appear to be of value to study a drug for its effect on eradicating the virus completely with survival of the culture, as well as for its mechanism of suppressing virus propagation in infected cultures. In this paper, we showed that while single drug administration was not enough to eradicate virus, adequate drug combinations could eradicate it from infected cells and save the cultures from final death. Many reports describe halogenated deoxyuridines as potent inhibitors of synthesis of DNA viruses: vaccinia(1,2,3,4,5), herpes simplex(2,6,7), adeno(3,8,9,10), pseudorabies(11), infectious canine hepatitis(12), and varicella(13); but none of them reports final eradication of the viruses. Kaufman and Maloney(5) reported that 5-iodo-deoxyuridine could eliminate vaccinia and herpes simplex viruses from tissue culture cells by the criteria of failure to detect virus, CPE and viral antigen in the cultures, but they did not describe the fate of the infected cultures after removing the drug. In our experiments, as in theirs, 5-BUDR, while continued in the medium, showed complete inhibition of virus induced CPE and virus propagation until 10 to 14 days after vaccinia infection. But we found that 5-BUDR did not completely eradicate the virus. Virus propagation and CPE occurred even in the presence of the drug during longer incubation periods. The other potent anti-vaccinia drugs studied in this laboratory, 5-me-TP and NOF, were also found to be incapable of eradicating the virus when they were used separately.

These experiments suggest that combinations of drugs which are able to eradicate the virus and save cultures from the progression of viral infection may be effective because of attack at both DNA and protein sites of virus synthesis. The fact that combinations of 5-me-TP and NOF, both of which may act only on protein synthesis, did not eradicate virus substantiates this suggestion.

Antiviral activity of 5-me-TP against T2 bacteriophage was reported by Cohen and Fowler(14), but we found it not effective against adeno type 12. Since both viruses respond to DNA interference from 5-BUDR, they are differentiated by their responses to the inhibitor of protein synthesis, 5-me-TP.

It is not possible, of course, to know from this study the fate of the individual infected cells, but it is encouraging to observe that survival of an infected culture has been made possible by an appropriate combination of compounds which singly fail to produce this effect.

Summary. Complete eradication of vaccinia virus and recovery of normal cells from the infected cultures of HeLa cells by treatment with drug combinations has been described. Combinations of 5-methyltryptophan and 5-bromodeoxyuridine or noformicin and 5-bromodeoxyuridine, which kept cultures nearly static without increase or decrease of cell population, were most adequate for this purpose. The recovered cells showed no immunity against vaccinia reinfection. Single drug administration could not eradicate the virus and could not save the infected cultures from final death. The eradication activity of the combination was doubtful against adeno type 12 virus.

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Some Factors Influencing Urinary Excretion of 3-Hydroxyanthranilic Acid. (29415)

HARRY SPIERA* AND CHARLES L. CHRISTIAN

Department of Medicine, Columbia University, College of Physicians and Surgeons, and Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York City

The increased excretion of 3-hydroxyanthranilic acid (3HAA) by a significant percentage of patients with rheumatoid arthritis has been reported by a number of independent investigators(1-3). This increased excretion has not been found in patients with other forms of arthritis or other types of disease, either infectious, granulomatous, inflammatory, degenerative, or neoplastic, with the exception of bladder carcinoma. 3HAA is an intermediary in the metabolism of the essential amino acid tryptophan by way of the kynurenine-nicotinic acid pathway(4). As far as is known, tryptophan is the only precursor of 3HAA(5). The cause for the increased excretion is not known. Prior studies have indicated that differences in dietary tryptophan intake are not responsible for the variations in 3HAA excretion(3). Among the possible mechanisms for the increased excretion are:

(1) An increased rate of gamma globulin turnover has been noted in some patients with rheumatoid arthritis(6,7). Since gamma globulin is relatively rich in tryptophan(8, 9), this increased turnover might lead to an increased production of 3HAA.

(2) Patients with rheumatoid arthritis ingest large quantities of salicylates. It has been shown that 3HAA oxidase, the enzyme responsible for 3HAA breakdown, is inhibited by salicylate *in vitro*(10). Therefore, it is possible that this inhibition occurs *in vivo* and thus prevents the breakdown of 3HAA.

(3) Patients with rheumatoid arthritis might, for reasons unknown, preferentially shunt tryptophan into the kynurenine pathway.

Whether these postulated mechanisms could indeed cause increased excretion of 3HAA was studied in a series of animal experiments.

Materials and methods. Six male white rabbits, each weighing approximately 2 kg, were maintained in metabolic cages on a standard diet and *ad lib* fluid intake. Urines were collected for consecutive 72-hour periods in the presence of concentrated sulphuric acid. After 2 control periods of 72 hours each, the rabbits were injected at 3-day intervals with formalin-killed *E. coli* as described by Abruzzo and Christian(11). Beginning the ninth week, the rabbits were injected with the bacteria daily. The animals were bled weekly. *E. coli* agglutinins and rheumatoid factor-like anti-gamma globulin antibodies were determined as previously described(11).

Another group of 6 similar rabbits were

* Fellow, Arthritis and Rheumatism Foundation.