Differential and Specific Inhibition of ECHO Viruses by Plant Extracts.* (25424)

NORMAND R. GOULET,† KENNETH W. COCHRAN AND GORDON C. BROWN
Dept. of Epidemiology and Virus Laboratory, School of Public Health, Univ. of Michigan, Ann Arbor

Although recent reviews(1-5) present many examples of inhibition of virus growth in particular complexes of host cell and virus, the chemotherapy of virus diseases remains a special problem. In this connection Bawden(3) observed that "a high degree of specificity, comparable with that possessed by viruses themselves, will be required from a substance that it should prevent the production of one kind of nucleoprotein while not interfering with others sufficiently to damage the synthetic mechanism of the host irreparably." That such specificity can be achieved was demonstrated in the present investigation through the use of plant extracts, some of which were found active against poliovirus in mice(6). Through the use of several ECHO viruses and plant extracts in tissue cultures of monkey kidney cells cytotoxicity of a susceptible virus was inhibited not only without damage to the host cell but also with preservation of the potentiality for responding to several other viruses.

Materials and methods. Monkey kidney cell cultures. Procedures for growth and maintenance of cultures were essentially similar to those reported by Youngner(7,8), modified by use of lactalbumin medium introduced by Melnick(9), and further modified by substitution of calf serum for horse serum. Buffering of medium was accomplished by reinforcement of bicarbonate and phosphate with 0.01M "Tris" (trishydroxymethylaminomethane.) Penicillin (100 units/ml), streptomycin (0.1 mg/ml) and mycostatin (100 units/ml) were incorporated into all cultures. *Viruses.* Thirteen Enteric Cytopathogenic Human Orphan (ECHO) viruses were employed, ECHO–1 through –9, and –11 through –14. These viruses were originally obtained from Dr. J. L. Melnick, Baylor University. Other viruses used included poliovirus type 1, Mahoney strain; Coxsackie B-1, Conn.–5 strain; and vaccinia, Michigan Dept. of Health. Virus was estimated by determining limiting dilution which could initiate infection in 50% of cultures inoculated. The 50% endpoints were calculated by the method of Reed and Muench(10). Virus inocula ranging 32 to 320 tissue culture 50% infectious doses ($TCID_{50}$) were employed in all tests, unless otherwise specified. Hyperimmune anti-ECHO serum was obtained by extensive intravenous and subcutaneous inoculation of young rabbits using high titered monkey kidney tissue culture antigens. *Antiviral extracts.* A number of crude and semi-purified extracts from *Calvatia gigantea,* and other mushrooms and plants, some of which possessed antitumor activity(11,12), were prepared‡ as listed in Table I. Extracts were assayed for toxicity prior to use in virus inhibitory studies. Certain gross cytological changes, such as distortion, pyknosis, swelling or sloughing, were the conditions associated with toxicity to the cultures. Only non-toxic dilutions were employed in the virus inhibitory studies. Except where stated, all cultures were routinely pretreated with extracts for 24 hours at 37°C prior to infection with virus. Antiviral activity was evaluated on the basis of delay, decrease, or prevention of characteristic virus-induced cytopathology when treated cultures were compared to appropriate controls. Effectiveness of these substances was judged by a therapeutic index(13) calculated from the ratio of lowest non-toxic dilution of the substance to maximum viral inhibitory dilution. For example, the therapeutic index of 28.8 for M2 *vs.* ECHO's 4 and 11 is calculated from

* This investigation was aided by grant from National Fn. and done in partial fulfillment of requirements for Ph.D. degree by senior author.

† Present address: Merck Sharp & Dohme, West Point, Pa.

‡ These extracts were prepared and furnished by the late Dr. E. H. Lucas, Dept. of Horticulture, Mich. State University.

TABLE I.

No.	Source and extraction
M1	*Boletus edulis,* fruiting body, chromatographic fraction of ethanol precipitate
2	*Calvatia gigantea,* #644, fruiting body, aqueous extract
3	*Calvatia gigantea,* #644, fruiting body, aqueous pH 6.0
4	*Calvatia gigantea,* #644, fruiting supernatant from ethanol precipitate
5	*Idem,* #643
6	" , #673
7	*Rumex acetosa,* 1:3 aqueous extract of leaves
8	*Hypericum* sp., 1:3 aqueous extract of whole plant
9	*Eleocharis dulcis,* 1:4 aqueous extract of corms
10	*Agaricus placomyces,* 1:2 aqueous extract of ethanol-treated sporophore
11	*Calceolaria crenatiflora,* 1:3 aqueous extract of flowers
12	*Ribes hirtellum,* 1:1 aqueous extract of ethanol-treated fruit
13	*Coprinus micaceus,* ethanol extract of sporophore
14	*Cattleya* sp., ethanol extract of flower
15	*Cattleya* sp., aqueous extract of residue from M14
16	*Calvatia gigantea,* #642, filtrate of blended mycelia and culture broth
18	*Polygonum persicara,* 1:5 aqueous extract of inflorescence, boiled

the ratio of 1/100, the lowest non-toxic dilution, to 1/2880, the maximum viral inhibitory dilution.

Results. Antiviral effectiveness of plant extracts. Results of testing 17 plant extracts against 13 of ECHO viruses are given in Table II. Some extracts showed differential inhibitory activity to a marked degree against some ECHO viruses, and had potential significance. M2, an aqueous extract of the fruiting body of a strain of *Calvatia gigantea,* the giant puffball, displayed significant activity against only ECHO 4, 9, and 11 at concentrations of 1:2880, 1:1200, and 1:2880 respectively. M4, an aqueous extract of the residue remaining from ethanol precipitation of the same species exhibited activity at concentrations of 1:960 and 1:1920 against ECHO 7 and 8 respectively. M14, prepared by ethanol extraction of the flowers of a species of *Cattleya,* exhibited activity at a concentration of 1:480 against ECHO 2. Other extracts were less effective when tested against other ECHO viruses.

Activity of M2 vs. ECHO-4 and 11. In view of the outstanding antiviral effectiveness of M2 against ECHO viruses 4 and 11, it was

TABLE II. Antiviral Activity of Plant Extracts *vs* ECHO Viruses.*

Extract	Lowest non-toxic dilution	Virus inhibited and therapeutic index†
M 1	1:80	E-12, *4*
2*	1:100	E-4, *28.8;* E-7, *4;* E-9, *12;* E-11, *28.8;* E-13, *6*
3*	1:120	E-7, *2;* E-9, *4;* E-13, *4*
4	"	E-1, *4;* E-2, *4;* E-3, *6;* E-4, *4;* E-5, *2;* E-7, *8;* E-8, *16;* E-9, *3;* E-11, *3;* E-12, *2;* E-13, *2*
5	1:10	E-2, *2*
6	1:120	E-3, *3;* E-7, *6;* E-8, *6;* E-12, *6*
7	1:100	None
8	"	E-1, *2;* E-12, *6*
9	1:200	E-6, *3;* E-12, *3*
10	1:100	E-1, *4;* E-11, *6;* E-12, *4*
11	"	E-1, *4;* E-6, *2;* E-9, *3*
12	"	E-9, *3;* E-13, *2*
13	"	E-11, *3;* not tested *vs* E-4, E-8, E-9, E-12, E-13
14	1:10	E-2, *48;* E-8, *4*
15	1:160	None
16	"	E-1, *2;* E-4, *2;* E-6, *2;* E-7, *2;* E-8, *2;* E-12, *2;* E-13, *4*
18	1:200	E-2, *3;* E-9, *6;* E-12, *4;* E-13, *4;* not tested *vs* E-1, E-3, E-5, E-6, E-7, E-8

* ECHO viruses tested 1-9, 11-13; E-14 tested with M2 and M3 only.

† Susceptible virus followed by therapeutic index in italics, therapeutic index was calculated as lowest non-toxic dilution over maximum inhibiting dilution, *i.e.* 1:100/1:2880 = 29.

TABLE III. Inhibition of ECHO Viruses 4 and 11 by M-2 Extract under Varying Conditions of Treatment and of Strength of Virus Inoculum.

Time of M-2 treatment	Virus $TCID_{50}$'s	Therapeutic index ECHO-4	ECHO-11
24 hr before virus	10,000	14.4	
Idem	3,200		9.6
"	1,000	28.8	28.8
"	100	14.4	"
With virus	"	"	
1 hr after virus	"	3.6	

of interest to explore its inhibitory activity more extensively by altering amount of virus and time of treatment. Antiviral activity of M2 varied depending on time of addition and potency of virus inoculum. These results are presented in Table III. Pretreatment of cultures with M-2 for 24 hours completely inhibited ECHO–4 virus up to and including 1000 $TCID_{50}$. Its effectiveness decreased against a challenge of 10,000 $TCID_{50}$ even with 24 hours pretreatment, and when added simultaneously with virus. Further reduction in effect occurred if treatment was withheld for 1 hour after infection of cultures with 100 $TCID_{50}$. Similar results were obtained with M4 against ECHO–11. The possibility that M2 might exert a direct action against free virus was investigated. One aliquot of ECHO 4 was treated with M2 (final concentration, 1:20) while 2 other aliquots each received equal volumes of normal maintenance solution. One of the latter was stored at 4°C and the other 2 aliquots incubated at 37°C for 24 hours. When the 3 samples were titrated for virus content, the titers were 10^{-5}, 10^{-5}, $10^{-4.5}$ for the 4° control, 37° control, and M2 treated samples, respectively. The same procedure was followed for ECHO–11 virus and the titers obtained were 10^{-4}, and $10^{-3.5}$ respectively. No evidence of direct antiviral action was obtained.

Specificity of antiviral activity. Since M2 inhibited selectively the cytopathologic effect of considerable amounts of ECHO viruses 4 and 11, we explored the specificity of this inhibition. Sets of 6 cultures were pretreated with 1/120, 1/240, 1/480, or 1/960 dilutions of M2 for 24 hours at 37°C. The cultures were then divided into 5 groups. Three of the groups were exposed to 100 $TCID_{50}$ of ECHO 4 and 2 groups were exposed to 100 $TCID_{50}$ of ECHO 11. Forty-eight hours later and 24 hours prior to appearance of complete cytopathology in virus control cultures, the fluids were removed, the cultures washed once, and fresh drug added to all treated cultures. At this time large doses of either ECHO–4 (10,000 $TCID_{50}$), ECHO-11 (3,200 $TCID_{50}$) or ECHO–1 (100,000 $TCID_{50}$) were added to one of each group of cultures previously exposed to either ECHO 4 or 11 virus. Data are presented in Table IV. It can be seen from results with the drug control cultures in group 6 that all dilutions of M2 tested protected the cultures against cytopathology from 100 $TCID_{50}$ of either ECHO 4 or 11 viruses for at least 72 hours after initial virus exposure and for the 1/120 dilution for 120 hours. There was also complete protection with stronger concentrations of M2 against re-exposure to those agents even when larger doses of ECHO 4 or 11 were employed (Groups 1, 2, 4, 5). When smaller quantities of M2 (1/480 and 1/960) were employed, cytopathology was prevented on the first exposure to virus, but not when larger doses of virus were added on re-exposure. In all these instances the virus which finally broke through the inhibitory mechanisms was recovered and typed, and was always the second virus. However, addition of ECHO–1 virus to cultures previously protected against type 4, resulted in infection of all the cultures.

An additional experiment was designed to complement above findings. M2 in 1/200 dilution was used to treat cultures which were then exposed to 100 $TCID_{50}$ of ECHO–11 virus, and 48 hours later were re-exposed to 0.1 ml of one of the following: 10^{-1} dilution of ECHO 1 through 7, and 11, as well as poliovirus 1, Coxsackie B1, and vaccinia virus. The challenge doses varied from 100 to 100,000 $TCID_{50}$. In this experiment the fluids were not changed prior to second virus exposure. Cytopathology was prevented when cultures were re-exposed to ECHO 4 or 11, but was complete when cultures were secondarily exposed to a virus not affected by M2. (Table V).

Antiviral activity of M2 in vivo. Demonstration of activity against an ECHO virus *in vivo* is complicated by the lack of readily de-

TABLE IV. Inhibition of Cytopathology* of M-2 Treated Monkey Kidney Cultures after Double Virus Exposures.

Group No.	Cultures pretreated 24 hr with M-2	1st exposure (100 $TCID_{50}$) †	2nd exposure, 8 hr after 1st $TCID_{50}$	Cytopathology Hr after 1st virus exposure 72	96	120	Type of ECHO virus recovered
1	1/120	ECHO-4	ECHO-4	—	—	—	nd‡
	1/240	"	(10,000)	—	+		nd
	1/480	"		—	+		4
	1/960	"		+			4
2	1/120	"	ECHO-11	—	—	—	nd
	1/240	"	(3200)	—	—	—	nd
	1/480	"		—	+		11
	1/960	"		+			11
3	1/120	"	ECHO-1	+			1
	1/240	"	(100,000)	+			1
	1/480	"		+			1
	1/960	"		+			1
4	1/120	ECHO-11	ECHO-4	—	—	—	nd
	1/240	"	(10,000)	—	+		4
	1/480	"		—	+		4
	1/960	"		+			4
5	1/120	"	ECHO-11	—	—	—	nd
	1/240	"	(3200)	—	—	—	nd
	1/480	"		—	+		nd
	1/960	"		+			11
6	1/120	ECHO-4 or -11		—	—	—	nd
Drug control	1/240	"		—	—	+	nd
cultures	1/480	"		—	+		nd
	1/960	"		—	+		nd
Virus controls		"		+			nd

* Designations (—) (+) represent protection and degeneration of cultures.
† Cultures washed once and fresh M-2 added with virus.
‡ Not done.

tectable disease in most laboratory animals (9). However, reports that use of certain antiviral antibiotics altered the immune response to an inoculated virus provided one approach to this problem(14,15). In preliminary experiments, 3 intraperitoneal injections of ECHO virus were sufficient to produce detectable levels of serum antibodies in mice. Accordingly 3 inoculations of 0.5 ml of undiluted, infected tissue culture fluid were given to mice at weekly intervals, one group receiving ECHO–4 virus and the other ECHO–11. Certain mice were treated with 0.01 ml/g of 4-fold diluted M2, which was given intraperitoneally the day before, the day of, and the day after inoculation of antigen. This triad of treatments was repeated with each injection of antigen, while other mice received only M2 and no antigen. At the end of 4 weeks all mice were bled and their sera assayed for antibody content in a monkey kidney tissue culture neutralization test. The results showed that M2 did inhibit formation of antibody since treated mice developed lower titers, 1:6 and 1:12 to ECHO viruses 4 and

TABLE V. Cytopathology of M-2 Treated Monkey Kidney Cultures after Double Virus Exposures.

1st exposure virus,* 100 $TCID_{50}$	2nd exposure virus, 0.1 ml of 10^{-1}	Cytopathology† Hr after 1st virus exposure 72	96	120
ECHO-11	ECHO-1	+		
"	2	+		
"	3	+		
"	4	—	—	—
"	5	+		
"	6	+		
"	7	+		
"	11	—	—	—‡
"	Polio	+		
"	Coxsackie	+		
"	Vaccinia	+		
"	Untreated controls	+		

* 24-hr pretreatment with M-2.
† Cytopathology indicated by — or + representing protection or degeneration.
‡ Evidence of occasional plaques.

11, respectively, than did mice inoculated only with virus, whose respective titers were 1:16 and 1:64. Mice receiving only M2 developed no detectable antibodies.

Discussion. It has been amply demonstrated that viruses respond differently to various chemicals(1-5). In the present investigation a number of ECHO viruses were studied to determine the character of their responses to chemical agents. Differential inhibition with significant individual specificity against certain of these viruses was found in extracts from the mushroom *Calvatia gigantea* (M2 and M4) and from a species of *Cattleya* orchid (M14). In monkey kidney cell cultures M2 inhibited the cytotoxicity of both ECHO 4 and 11 without influencing the cytopathogenicity of several apparently related viruses. Since it was demonstrated that the maximum concentration of M2 employed had no pronounced, direct inactivating effect on these viruses, that possibility may be eliminated in explaining its antiviral activity. No direct evidence was obtained that these preparations specifically inhibit virus multiplication but the available data do indicate that M2 acts cellularly to inhibit the cytopathogenic effect of ECHO viruses 4 and 11 and that it does not block processes essential either to subsequent cytopathogenesis by other selected viruses or for maintenance of host cell vitality. Since both ECHO 4 and 11, individually or consecutively in the same culture, can be inhibited by M2 one can also deduce that they share similar if not identical cytopathogenic processes. These results also indicate that among these presumably closely related enteroviruses, reactions involved in the infectious processes of some are sufficiently distinctive to reveal marked differences in their individual vulnerabilities to chemical agents.

Summary. 1) Comparison of antiviral activity of several plant extracts against several ECHO viruses revealed that M2, prepared from a strain of *Calvatia gigantea* significantly inhibited only ECHO 4 and 11; while M4, a different preparation from the same strain, inhibited only ECHO 7 and 8; and M14, from a species of *Cattleya,* inhibited ECHO 2. The effectiveness of M2 *in vitro* was greatest when the cells were pretreated and when the virus inocula did not exceed 100 $TCID_{50}$. 2) Specificity of the antiviral action of M2 was demonstrated by inhibition of one M2-susceptible virus followed in the same culture by growth of a second M2-nonsusceptible virus. Inhibition continued, however, if the cultures were reinoculated with a second M2-susceptible virus. 3) Activity of M2 was demonstrated *in vivo* by its ability to suppress the antigenicity of an M2-susceptible virus. 4) The results demonstrate strikingly the capacity of the materials to prevent infection of susceptible cells by specific viruses although the cells retain their physiologic competence to support growth of other viruses and the virus is not directly affected by the inhibitory material. Obviously the effect must be directed to modification of reactions essential to viral development but not injurious to functional integrity of the cell.

1. Matthews, R. E. F., Smith, J. D., *Adv. in Virus Research,* 1955, v3, 49.

2. Hurst, E. W., Hull, R., *Pharmacol. Rev.,* 1956, v8, 199.

3. Bawden, F. C., *Adv. in Virus Research,* 1954, v2, 31.

4. Francis, T., Jr., *Poliomyelitis,* 4th Internat. Poliomyelitis Conf., 1958, p361, J. B. Lippincott Co., Philadelphia.

5. Horsfall, F. L., Jr., Tamm, I., *Ann. Rev. Microb.,* 1957, v11, 339.

6. Cochran, K. W., Lucas, E. H., *Antibiot. Annual* 1958-1959, 104.

7. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

8. ———, *J. Immunol.,* 1954, v73, 392.

9. Melnick, J. L., *Ann. N. Y. Acad. Sci.,* 1955, v61, 754.

10. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

11. Lucas, E. H., Ringler, R. L., Byerrum, R. U., Stevens, J. A., Clarke, D. A., Stock, C. C., *Antibiot. and Chemother.,* 1957, v7, 1.

12. Lucas, E. H., Byerrum, R. U., Clarke, D. A., Reilly, H. C., Stevens, J. A., Stock, C. C., *Antibiot. Annual,* 1958-1959, 493.

13. Kramer, P. E., Robbins, M. L., Smith, P. K., *J. Pharmacol. Exp. Therap.,* 1955, v113, 262.

14. Powell, H. M., Culbertson, C. G., McGuire, J. M., Hoehn, M. M., Baker, L. A., *Antibiot. and Chemother.,* 1952, v2, 432.

15. Shope, R. E., *J. Exp. Med.,* 1953, v97, 639.

Received August 14, 1959. P.S.E.B.M., 1960, v103.