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Effects of Cortisone on *in vitro* Viral Infection. I. Inhibition of Cytopathogenesis by Polio, Rabies and Yellow Fever Viruses in Human Embryonic Cells.* (30124)

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The action of steroid hormones on *in vitro* infection of cells by viruses has been studied with different systems(1-8). Contrasting results have been reported for action of cortisone, including protection against cytopathic effect coupled with increased virus production (4), enhanced cytopathic effect accompanying increased virus release(5), prolonged survival of chick embryos associated with initial inhibition of viral synthesis but final increase in virus yield(2,3), and reduction of virus titers revealed by cortisone-treated cells(6-8). It was therefore of interest to reexamine the effects of cortisone on *in vitro* virus infection with the human diploid cell strains now available(9). This paper reports some effects on cytopathogenesis induced by 3 different viruses.

Materials and methods. Cell-culture systems. The following strains of normal human fibroblasts were used in the experiments: the WI-38 human diploid cell strain(9) derived from lung tissue of normal human embryo and used at the 20th culture passage level, the 418 strain derived from lung tissue of normal human female embryo and used at the level of the 9th culture passage, and the HLT strain from normal human embryo, which was used at the 30th subculture level. The 418 strain was started by the procedure described by Hayflick and Moorhead(9). For purpose of this study, the HLT strain was initiated from tissue fragments explanted in medium with and without 2.5 μg/ml cortisone acetate (Vitamix Corp., Philadelphia, Pa.). Methods employed for the subculture of cell strains, or for preparation of monolayer cultures in tubes or in Petri dishes, followed exactly the techniques described in detail elsewhere(9), except that the 418 strain was subcultured by 1:3 rather than 1:2 splits. Cells were dispersed for subculture by treatment with 0.25 mg/ml trypsin in Earle's solution. For treatment of cells during subculture, cortisone was added to medium to a concentration of 2.5 μg/ml, except as noted.

Viruses. The 17 D strain of yellow fever virus, 230th chick-embryo passage in the form of lyophilized suspension of chick embryonic tissues, was used as inoculum to start the cell-culture passage series described here. The CHAT strain of type 1 poliovirus (11) adapted to grow in human diploid cell strains was used for plaque-count experiments. The CVS (received from Nat. Inst. Health, Bethesda, Md.) and HEP-Flury [chick embryo adapted(10)] strains of fixed rabies virus were used in the form of infected mouse brain and infected chick embryo suspensions, respectively.

Effects of virus infection on cells. Cytopathic effects were determined by daily microscopic examination of cultures until division for subculture or observation of complete lytic destruction. Some cultures were stained by May-Grünwald Giemsa stain be-

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TABLE I. Effect of Cortisone on Response of Human Embryonic Lung Cells to CHAT-Strain Type 1 Poliovirus.

Exp No.	No. of cell passages in presence of cortisone before infection	Exposure of virus to cortisone before seeding	Plaque count per log virus dilution: 4.0	5.0	6.0	Virus titer PFU/ml,* log
1	none	—	conf†	49	19	7.3
	only during exposure to virus	—	conf	56	12	7.1
2	none	—	conf	68	32	7.5
	6	—	62	21	4	6.6
3	none	—	conf	conf	51	7.7
		4 hours	conf	conf	38	7.6
	6	—	38	7	4	6.0
		4 hours	42	12	7	6.2
4	none	—	conf	conf	24	7.4
	15	—	7	0	0	4.8
5‡	none	—	conf	56	6	6.8
	15	—	0	0	0	<4.0

* PFU = plaque-forming units; titer calculated from plaque counts given.
† conf = plaques confluent.
‡ HLT cell cultures were used; in all other experiments the WI-38 cell strain was employed.

fore examination. Plaque formation by poliovirus was determined with cultures in plastic Petri dishes as described by Dulbecco(12); for counting plaques, cultures were stained with neutral red 4 days after infection. For immunofluorescence analysis, cultures were stained directly by the technique of Coons and Kaplan(13) as applied by Goldwasser *et al*(14). Burro-antiserum-globulin conjugate was obtained from the National Rabies Laboratory, Atlanta, Ga. At least one thousand cells were counted for assessment of the proportion exhibiting specific fluorescence. Mouse infectivity of virus was determined by intracerebral inoculation of 18-23-day-old Swiss albino mice with serial decimal dilutions of infected-cell culture medium. Inhibitor production by 17 D virus-infected cell cultures was tested by heating supernatant medium to 56°C for 30 minutes to inactivate virus, adding medium in varied amounts to strain 418 cell cultures, and exposing these cultures after 24 hours to a 10^{-4} dilution of CHAT virus.

Results. Effect of cortisone on cellular response to poliovirus. To test the effect of cortisone on cellular susceptibility to poliovirus infection, WI-38 and HLT cells were propagated serially in culture with Eagle's basal medium supplemented with 10% (v/v) calf serum, in 2 series. The medium used for one series of each cell strain contained 2.5 μg/ml cortisone acetate. There was no difference in the number of plaques yielded by WI-38 cells of the first passage, when cortisone treatment was confined to the period of exposure of cells to virus (Table I). After 6 subculture passages in medium with and without cortisone, treated cells yielded fewer plaques than did cells passed in cortisone-free medium (Table I). Cells subjected to at least 15 subculture passages in medium containing cortisone exhibited decidedly fewer (Table I) and smaller plaques than WI-38 or HLT cells that were not so treated. Incubation of CHAT virus in cortisone-supplemented medium at 37°C for 4 hours before application to cells did not affect either the number or size of plaques obtained, in comparison with those produced by virus incubated in cortisone-free medium. The inhibitory effect of cortisone on virus multiplication appeared to be exerted on the cells rather than virus, and its manifestation required repeated passage of cells in cortisone-supplemented medium.

Effect of cortisone on cellular response to rabies virus. HLT-strain human embryonic fibroblast cultures were infected with rabies virus of the CVS or HEP-Flury strain. Cells of the 2 virus-infected sets were propagated serially in 2 paired subsets, one subset of each pair being fed with medium containing 2.5 μg/ml cortisone and the other subset fed with cortisone-free medium. At passage levels 1, 4, 6, 10 and 12, cellular response to

TABLE II. Effect of Cortisone on Response of HLT Human Embryonic Lung Cells to Rabies Virus.

Virus strains	Cellular effect	No. of cell passages in presence (+) or absence (0) of cortisone: 1		4		6		10		12	
		0	+	0	+	0	+	0	+	0	+
CVS	% IF*	5	trace	90	5	100	5	100	5	100	5
	CPE†	0	0	1‡ (12)§	0	1 (15)	0	2 (12)	0	comp‖	0
	LD_{50}¶	>5.5	4.0	3.9	1.6	4.3	2.5	N.D.**	N.D.	N.D.	N.D.
HEP-Flury	% IF	3	0	20	0	50	0	100	0	100	0
	CPE	0	0	trace (14)	0	1 (15)	0	2 (15)	0	comp	0

* % IF = No. of specifically fluorescent cells per 100 cells examined.
† CPE = degree of cytopathic effect graded from trace (few areas of cell rounding) to complete (total disruption of cell sheet).
‡ Grade 1 cytopathic effect.
§ Figures in parentheses denote day when degree of cytopathic effect was observed.
‖ Comp = complete cytopathic effect.
¶ Titer of infectivity for mice, logarithms.
** N.D. = not done.

the initial infection was assessed by observing the degree of cytopathic effect as revealed by cell rounding and failure of sheet formation, by counting the number of specifically reacting cells indicated by immunofluorescence staining to detect viral antigen in cells and, with CVS-virus-infected cells, by titrating in mice the virus present in the supernatant medium of the cultures. Presence of cortisone in culture-passage medium suppressed the cytopathic effect of both strains of rabies virus on the HLT cells (Table II). After 4 and 6 passages, there was less virus in the medium of cortisone-treated CVS-virus-infected cultures than in medium from control cultures; with either virus strain, the spread of infection through the cultures as shown by immunofluorescent stain was inhibited by cortisone treatment (Table II, Fig. 1).

Effect of cortisone on cellular response to yellow fever virus. Human cells of the 418 strain were exposed to 17 D-strain yellow fever virus at a level equivalent on the average to one virus particle per 10 cells. A cytopathic effect, appearing first in the culture on the 4th day after infection, progressed to complete lysis of the cells by the 9th day. Addition of 2.5 μg/ml cortisone to the medium 24 hours after infection of the culture with virus completely inhibited the cytopathic effect, without reducing the yield of infectious virus (Table III). Following this observation, infected cells grown in the presence of cortisone were dispersed with trypsin and sub-cultured. Again no cytopathic effect was noted (Table III, passage 2). Such subculture was repeated every 3-10 days until the 10th passage was attained. This passage level represented maintenance of the cultures for 120 days after initial infection with yellow fever virus. During this time the infected cells grew well, without gross alteration of morphology. At 3 subculture levels of the series (2, 4 and 10), as well as the subcultures maintained with cortisone-supplemented medium other companion cultures were prepared from the infected trypsin-dispersed cells. This second set of cultures was fed with cortisone-free medium and incubated for observation of cytopathic effect. In these cultures transferred to cortisone-free medium, a complete lysis of the cells occurred by the 9th to 12th day after transfer (Table

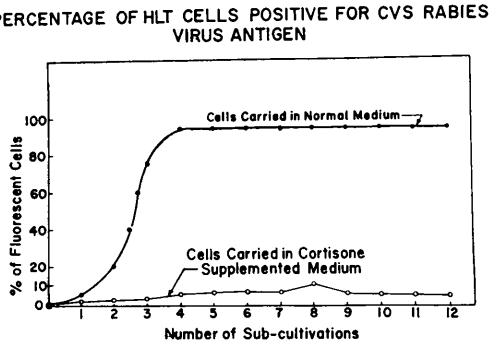

FIG. 1. Proportion of infected cells in HLT human-embryonic cell cultures inoculated with CVS-strain rabies virus and subcultivated serially in cortisone-containing and cortisone-free medium, as revealed by specific immunofluorescence.

TABLE III. Effect of Cortisone on Response of 418-Strain Human Embryonic Lung Cells to 17 D-Strain Yellow Fever Virus.

Cell passage in cortisone medium		Effect of virus infection on cells:			
		Passed in cortisone medium		Transferred to cortisone-free medium	
Passage No.	Days after infection	CPE*	LD_{50}†	CPE	LD_{50}
None‡	4	+	4.0		
	9	comp§	N.D.‖		
1	4	0	3.8		
	9	0	N.D.		
2	14	0	>2.0	comp	>3.0
3	17	0	>2.0		
4	28	0	N.D.	"	N.D.
5	31	0	>3.0		
10	120	0	trace¶	"	2.6

* CPE = cytopathic effect recorded as 0 (no effect), + (cell rounding and disruption of cell sheet), or complete (comp) cell lysis after 9-12 days of incubation.

† Titer of infectivity for mice, logarithms.

‡ These cultures, infected in medium without cortisone, were companion to the passage 1 cultures infected in medium with cortisone; subcultures of the cortisone-passed cells at passages 2, 4 and 10 were divided to provide one set for continuation of the cortisone series and one set for feeding with cortisone-free medium and observation for cytopathic effect.

§ Comp = complete.

‖ N.D. = not done.

¶ Trace: 2 of 12 mice injected with undiluted cell-culture fluid died.

III). The yield of infectious virus from cultures cultivated for 120 days in the presence of cortisone was very low (trace), but other cells of this population produced virus ($10^{2.6}LD_{50}$) after their transfer to medium without cortisone.

Amplification of these experiments confirmed the previously noted inhibition of cytopathic effect of 17 D virus by exposure of cells grown in standard medium (Eagle's basal medium with 10% calf serum) to cortisone (5 μg/ml) following infection with virus (Table IV). Furthermore, a similar degree of inhibition of cytopathic effect was observed with cells grown during 4 culture passages in the presence of cortisone, before infection (Table IV). It may be noted, too, that the intensity of the cytopathic effect on cultures maintained in medium without cortisone after virus infection was unrelated to the pretreatment of these cultures with cortisone. The yield of virus did not seem to be affected by any of the treatments of the cultures.

To see whether production of interferon might be involved in the effect of cortisone on cellular response to virus infection, fluids were removed from the infected cultures which had been cultivated for 120 days in the presence of cortisone (cell passage 10, Table III). These fluids were tested for their effect on susceptibility of 418-strain cells to subsequent infection with CHAT-strain poliovirus. As shown by the results summarized in Table V, prior exposure of the cultures to medium from 17 D virus-infected cortisone-treated cells produced a marked inhibition of cytopathic effect of the poliovirus. This inhibition was not observed with cultures preexposed to medium from infected cells not treated with cortisone (other control experiments showed no effect of heating of the medium or of heated cortisone on response of human diploid cells to the poliovirus). It is important to stress the fact that in the first case (when interferon-like activity was observed) only traces of active virus were present originally in the tested fluids; in the latter case (where no inhibition was noted) an appreciable amount of virus was detectable before heating.

Discussion. Cortisone has been reported to both decrease(3,6-8) and increase(3-5) virus yield during *in vitro* infection, and both prevent(2,4,7,8) and accelerate(1,5) cytopathic effect of virus infection. Differences in host cells, viruses, and particularly cortisone dosage, and time of action may account for these contrasting findings. With the cortisone dosage employed by Holden and Adams(5), probably toxic, giant cells appeared in cultures. If the giant cells had facilitated virus multiplication, as has been reported(15), their presence would have complicated evaluation of cortisone action. In the present work a physiologic level of cortisone was employed. With this dosage, treated fibroblastic cultures of normal human embryonic origin exhibited much less severe cytopathic effect of infection with polio, rabies and yellow fever viruses than control cells not treated with cortisone. Titers of virus released by treated cells compared with untreated cells

were lower when treatment was prolonged through many cultural passages of the cells. As investigated with poliovirus infection by plaque analysis, cortisone did not affect virus or its adsorption, but was effective when cells were subjected to more than 6 cultural passages in its presence. Accumulation of infected cells during serial subculture of cells initially infected with rabies virus was inhibited by cortisone in the medium, as shown by the number of cells containing immunologically detectable virus, by the progression of cytopathic effect, and by the level of infectious virus found in the supernatant medium. Exposure of cultures to cortisone after infection with yellow fever virus inhibited cytopathic effect without reducing the titer of released virus significantly. Released yel-

TABLE IV. Effect of Cortisone on Response of WI-38 Human Diploid Cell Strain to 17 D-Strain Yellow Fever Virus.

Presence of cortisone in culture medium:				Degree of cytopathic effect on day:			
Before exposure to virus	After infection	Virus challenge multiplicity	Day 3 supernate virus: LD_{50}/.03 ml	3	5	7	10
None	None	No virus	—	0,0*	0,0	0,0	0,0
		.015	N.T.†	0,1	2,2	2,2	2,2
		.05	3.5	0,1	1,2	2,2	2,2
	Present‡	.015	N.T.	0,0	1,0	1,0	1,0
		.05	3.5	0,0	1,0	1,0	2,0
Present§	None	.015	N.T.	1,0	1,1	1,1	2,2
		.05	4.3	2,2	2,2	2,2	2,2
	Present‡	No virus	—	0,0	0,0	0,0	0,0
		.015	N.T.	0,0	0,0	0,0	0,0
		.05	3.0	0,0	0,0	0,0	0,0

* Responses for duplicate cultures, graded as 0 (no effect), 1 (patches of cell rounding), 2 (extensive areas of cell rounding and destruction), etc.

† N.T. = not titrated.

‡ Infected cultures were maintained for 10 days in the presence of cortisone at 5 μg/ml concentration.

§ Cells were kept for 4 consecutive transfers during 14 days in medium containing cortisone at 5 μg/ml concentration.

TABLE V. Test for Interferon-Like Activity in Fluids from Carrier Cultures of 418 Human Embryonic Cells Infected with 17 D Virus.

Source of fluid*		Cytopathic effect of CHAT poliovirus on indicator monolayers exposed previously to dilution of medium from cortisone-treated or untreated cultures: †		
From subculture	Cortisone in medium	Undiluted	1/2	1/10
10 C	Present	0‡	0	1
11 C	"	0	0	1
10	Absent§	3	4	4
Fresh Eagle's medium (poliovirus control)	"	4	4	4

* Strain 418 human embryonic lung cells were infected with 17 D yellow fever virus and transferred serially in cortisone medium for 120 days (Table III); 9th-passage cultures were split, and one subculture received cortisone medium (10 C) while the other received cortisone-free medium(10). A following subculture received cortisone medium (11 C). Fluids from the 3 cultures were heated to 56°C for 30 min to inactivate virus before testing for interferon-like activity.

† Uninfected monolayers of 418 cells were exposed to test fluids for 24 hr, then inoculated with a 10^{-4} dilution of poliovirus, to determine effect of exposure on susceptibility to poliovirus.

‡ Cytopathic effect graded from 0 (no effect) to 4 (complete cell destruction and monolayer disruption) according to degree and extent of cell rounding.

§ Cortisone was omitted from the medium on this subculture of the series otherwise maintained in cortisone medium.

low fever virus was reduced to a low level after 120 days of subculture of the infected cells, but both cytopathic effect and free virus reappeared on transfer of infected cells of the same subculture level to cortisone-free medium. While the need for prolonged exposure of cells to cortisone during cultural passage for inhibition of cytopathic effect coupled with decrease in virus production suggested a process of selection of variant cells resistant to cytopathic effect, the suggestion was not supported by the results with yellow fever virus. Here cellular susceptibility was recovered on removal of cortisone from culture medium, and an influence of cortisone on cellular response after infection was confirmed. All of the findings together suggested an effect of cortisone on cellular capacity to react to infection, which was cumulative but not selective. The effect might have resulted from such a mechanism as that of interferon production by cortisone-treated cells, or that investigated in a later report (16). This confirmation of an inhibitory effect of cortisone treatment on cytopathic response of cells to infection with the three viruses is of interest in relation to reported separate effects of cortisone on virus production and inflammation (2,3).

Summary. Plaque formation, observation of cytopathic effect, immunofluorescence and mouse inoculation were used variously to evaluate some aspects of cortisone acetate on infection of diploid human embryonic fibroblastic cultures with polio, rabies and yellow fever viruses. Cortisone did not affect poliovirus directly, but did inhibit plaque production when cells were subjected to many passages in its presence. Accumulation of cytopathic effect and spread of virus in cultures infected with rabies virus before serial subculture both were inhibited by cortisone in passage medium. Cortisone exerted a similar cumulative effect on virus production by cells infected with yellow fever virus, and an immediate inhibition of cytopathic response, but both virus production and cytopathic effect returned on removal of cortisone from medium. The findings suggested a cumulative but non-selective effect of cortisone on cellular capacity to respond to virus infection.

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