A NON-TRANSMISSIBLE CYTOPATHOGENIC EFFECT OF INFLUENZA VIRUS IN TISSUE CULTURE ACCOMPANIED BY FORMATION OF NON-INFECTIOUS HEMAGGLUTININS*

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A number of viruses pathogenic for man have been adapted to grow in cultures of cells derived from a cervical carcinoma (strain HeLa) (1, 2). Attempts to pass influenza viruses serially in these cells have failed thus far. Yet, in the present studies, a direct cytopathogenicity of these agents could readily be demonstrated provided sufficient quantities of virus were added to the cultures. No evidence was obtained of production of infectious virus in HeLa cells, but considerable titers of hemagglutinins and of complement-fixing antigens became detectable in 12 to 24 hours. These results suggest that an "incomplete" reproductive cycle may take place in HeLa cells comparable to that observed by Schlesinger in cells of the central nervous system of mice, upon intracerebral injection of non-neurotropic strains of influenza virus (3). It is the purpose of this report to relate the findings.

Methods and Materials

HeLa Cells.—A strain of these cells was originally obtained from the Microbiological Associates, Inc., and used in the earlier experiments. Later on, certain changes were noted in the cultures exemplified by periodic stimulation of rapid growth and subsequent deterioration of cells. From survivors new cultures could readily be initiated only to undergo a similar course of events. Since these cells exhibited altered susceptibility to poliomyelitis and other viruses, a new line of HeLa cells was kindly supplied by Dr. W. F. Scherer. Essentially similar results with respect to influenza viruses were obtained with these 2 lines.

The methods employed for growing the cells were essentially similar to those described by Scherer (1).

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(*a*) *Preparation of Bottle Cultures.*—For bulk production, 16 oz. Blake bottles were seeded with approximately 4×10^6 cells suspended in 20 ml. of a medium containing 40 per cent human serum, 4 per cent acetone-extracted chick embryo extract, and 56 per cent of either Hanks's balanced salt solution or the maintenance solution (M.S.) of Scherer (4). After 3 to 4 days of incubation at 36°C., 50 per cent of the fluid was replaced by fresh medium of the same type. Following further incubation for a total of 1 week the cultures, as a rule, contained approximately 2×10^7 cells and were then employed for preparation of test tube or further bottle cultures, or directly for virus studies.

(*b*) *Preparation of Test Tube Cultures.*—Mature bottle cultures were treated in one of two ways depending on whether human or horse sera were to be used for the growth phase in the culture tubes. In the first case, the cells were scraped off the glass by means of a rubber policeman into the medium in which they had been growing and sufficient fresh medium, consisting of 30 per cent human serum and 70 per cent Hanks's solution, was added to give a concentration of about 10^5 cells per ml. Each test tube was seeded with 0.4 ml. of this suspension and incubated at 36°C. for 3 to 4 days. At this time, the cultures had developed sufficiently, the growth medium was removed, the cells were washed 3 times with 1 ml. volumes of Hanks's solution and finally, 0.9 ml. of maintenance medium was added, consisting of 10 per cent horse serum and 90 per cent M.S. If horse serum was to be employed in the growth medium the bottle cultures were drained and the cells were washed 3 times with 10 ml. of Hanks's solution. They were then scraped off into a medium containing 20 per cent horse serum and 80 per cent M.S. and the test tubes were seeded with 0.6 ml. of this suspension. After 2 to 3 days of incubation the medium was replaced by 0.9 ml. of maintenance medium (10 per cent horse serum and 90 per cent M.S.). This technic avoided the cumbersome washing of the cells in test tubes in order to remove antibodies the human sera may contain. The tube cultures were employed for virus studies usually within 1 week after the change to maintenance medium.

Propagation of Cells in the Presence of P^{32}.—The isotope in form of phosphate ions in dilute HCl was added following adjustment of the pH to neutrality in 50 μc. amounts to each bottle culture immediately after seeding. At the time of 50 per cent medium renewal the isotope withdrawn was replaced by the addition of 25μc. The cells obtained after maturation of the cultures were seeded again in bottles under the same conditions. After satisfactory sheets had formed, these cultures were ready for infection.

Influenza Viruses.—Three strains of influenza A virus (PR8, F99, and $L_2$47) and the Lee strain of influenza B were employed. Seeds were prepared by inoculation of 11- to 12-day old chick embryos by the allantoic route with 0.5 ml. of infected allantoic fluids diluted to 10^{-5} to 10^{-6}. After incubation of the eggs at 36 to 37°C. for 48 hours the allantoic fluids were collected, dialyzed in the cold room against 20 volumes of 0.01 M phosphate buffered saline solution of pH 7.2, distributed into ampuls and stored at −65°C. These stock preparations, after thawing, were diluted to the desired concentrations in maintenance medium prior to addition to tube cultures, usually 0.2 ml. being added. On occasion, native allantoic fluids were used as seeds with similar results.

Virus Assays.—The technics for titrations of infectivity and hemagglutinins have been fully described (5, 6). For the complement fixation tests serial 2-fold dilutions of the antigens were prepared in triplicate using 0.1 ml. volumes. To the respective series of dilutions were added 0.2 ml. of equal mixtures of (*a*) diluted complement (2 units) and optimally diluted guinea pig serum *vs.* PR8-V or virus antigen, which did not react with the soluble or S antigen; (*b*) complement and guinea pig anti-$L_2$47 serum, which cross-reacted only with PR8-S but not with PR8-V antigen; and (*c*) complement and saline solution. After primary incubation at 4°C. for 18 hours, 0.2 ml. of sensitized sheep red cells were added to each tube and the test was read after further incubation at 37°C. for 1 hour. The last dilution of antigen giving

a 3 or 4+ fixation of complement was taken as the endpoint. The results are expressed as antigen units per milliliter.

Other technical details are described in the text.

TABLE I

The Cytopathogenic Effect of Various Strains of Influenza Virus

Seed				Cytopathogenic effect													
				Dilution of seed													
				10^{0}			10^{-1}			10^{-2}			10^{-3}			10^{-4}	
				Day of incubation													
Experiment No.	Strain	EID_{50}/ml.	HA/ml.	2	4	6	2	4	6	2	4	6	2	4	6	2	4
		log	*log*														
1	PR8	9.9	3.4	4*	4	4	4	4	4	1	4	4	0	0	0	—	—
	F99	9.6	3.1	4	4	4	2	4	4	1	3	4	0	0	0	—	—
	$L_2$47	9.6	3.1	2	4	4	1	3	3	0	0	0	0	0	0	—	—
	Lee	8.1	3.1	3	4	4	2	4	4	1	3	4	0	0	0	—	—
	NAF‡	—	—	0	0	0											
2§	PR8	9.9	3.4	4	4	4	2	4	4	1	3	3	0	0	0	—	—
	F99	9.6	3.1	3	4	4	1	3	4	0	0	0	0	0	0	—	—
	$L_2$47	9.6	3.1	2	4	4	±	2	3	0	0	0	0	0	0	—	—
	Lee	8.1	3.1	2	4	4	1	4	4	0	0	0	0	0	0	—	—
	NAF‡	—	—	0	0	0											
3	PR8	10.0	3.7	—	—		3	4	—	2	3	—	0	1	—	1	±
4	PR8	—	3.4	—	4		—	4	—	—	3	—	—	1	—	—	±

* 4, all cells destroyed. ± to 3, intermediary stages of cellular destruction. 0, no cytopathogenic effect.

‡ Non-infected allantoic fluid.

§ Cells grown in human serum, all others in horse serum.

EXPERIMENTAL

The Effect of Influenza Viruses on HeLa Cells.—It was found that the addition of influenza virus to test tubes containing sheets of HeLa cells on maintenance medium led to the appearance of cytopathogenic effects of varying degrees within 12 to 96 hours depending on the concentration of the agent employed. The cytopathogenic effect was readily prevented by specific rabbit immune serum against the homologous but not against heterotypic virus. All four strains of influenza virus used showed similar effects (Table I). The cells acted upon first appeared rough and granular, then became rounded and shrunken and finally sloughed off the glass. It is seen in the table that the seeds could be diluted at most to 10^{-4} and often not more than to 10^{-2} in order

to produce some evidence of cellular destruction. The results were not strikingly different whether the cells had been grown initially in human serum (Experiment 2) or in horse serum (Experiments 1, 3, and 4) prior to maintenance on medium containing only horse serum. Thus at least in the order of 10^6 egg-infectious doses (EID_{50}) were required to yield *some* cytopathogenic effect and considerably more for *complete* destruction of the cells.

It was apparent that with the more dilute inocula often only partial degeneration was noted even after prolonged incubation. This indicated that the process was restricted to the cells originally infected by the seed virus. This suggestion is supported also by the results of experiments in which small doses of virus were inoculated at the time the test tube cultures were initially set up. From the non-infected cells eventually healthy cultures were obtained, although a longer time was required for the establishment of sheets than in the absence of virus, obviously on account of the reduction in viable cells. The cultures obtained under these conditions were readily destroyed when a second larger inoculum of virus was given at the time when sheets had formed. Thus, the cells surviving the first smaller dose of virus were not resistant to the cytopathogenic effect.

Attempts at Serial Passages of Influenza Viruses in HeLa Cells.—Repeated attempts at serial passage of influenza virus in HeLa cells by transfer of undiluted tissue culture medium as well as of cells have failed. Although cytopathogenic effects were seen up to the 3rd or 4th passage, they developed in succeeding passages after increasingly longer periods of incubation and the degrees of destruction decreased. It was apparent that these results were caused by the carry-over of some of the original seed virus which gradually became too dilute on passage to induce lesions, the 4th passage corresponding to a seed dilution of approximately $10^{-2.8}$. Passage series using diluted virus and media for transfers likewise failed.

Effect of Partial Inactivation of Virus on the Cytopathogenic Effect.—These results appeared to be comparable to the "toxic effects" of influenza virus when administered to experimental animals by routes other than the respiratory tract (8–11). The toxic effect is seen particularly in tissues which do not support complete cycles of viral multiplication and is obtained only after injection of large concentrations of virus. The toxic activity of the virus is somewhat less sensitive to inactivating agents such as ultraviolet light than the infectious property (9, 12). Indeed, virus preparations irradiated by ultraviolet light to the extent that they were no longer infectious but still retained interfering activity, still exerted some cytopathogenic effects (Table II, Experiment 1). However, the non-infectious preparations revealed lower titers of cytopathogenicity and the lesions developed late in the incubation period. If the virus preparations were exposed to ultraviolet light for increasingly longer periods of

time (Table II, Experiment 2) the cytopathogenic effect decreased further and, when the hemagglutinating activity of the virus became ultimately affected by the irradiation procedure, even the strongest concentration of the seed no longer caused destruction of cells. Similarly, when a virus preparation was heated at 37°C. for increasing periods of time its cytopathogenic effect de-

TABLE II

Cytopathogenic Effect of Ultraviolet-Irradiated Influenza Virus

Seed					Cytopathogenic effect													
					Dilution of seed													
					10^0				10^{-1}				10^{-2}				10^{-3}	
					Days of incubation													
Experiment No.	Strain	U.V.	EID_{50}/ml.	HA/ml.	1	3	4	5	1	3	4	5	1	3	4	5	3	4
		min.	*log*	*log*														
1	PR8	0	9.9	3.4	3	4	4	4	2	4	4	4	0	3	4	4	—	—
		3	<0.8	3.4	0	2	3	4	0	0	2	3	0	0	0	0	—	—
	F99	0	9.6	3.1	1	4	4	4	0	4	4	4	0	1	2	3	—	—
		3	<0.8	3.1	0	0	2	3	0	0	0	0	0	0	0	0	—	—
	$L_2$47	0	9.6	3.1	1	3	4	4	0	2	3	4	0	0	0	0	—	—
		3	<0.8	3.1	0	0	2	2	0	0	0	0	0	0	0	0	—	—
	Lee	0	8.1	3.1	0	4	4	4	0	4	4	4	0	1	3	4	—	—
		3	<0.8	3.1	0	0	3	4	0	0	3	4	0	0	0	0	—	—
2	PR8	0	9.0	3.4	—	4	4	—	—	4	4	—	—	2	3	—	±	1
		1	1.3	3.4	—	3	4	—	—	3	3	—	—	±	2	—	±	1
		3	<0.8	3.4	—	2	3	—	—	2	2	—	—	1	1	—	0	0
		6	—	3.4	—	1	1	—	—	1	1	—	—	±	±	—	0	0
		10	—	3.4	—	1	2	—	—	0	0	—	—	0	0	—	0	0
		15	—	3.4	—	±	±	—	—	0	0	—	—	0	0	—	0	0
		60	—	1.6	—	0	0	—	—	0	0	—	—	0	0	—	0	0
		120	—	<0.4	—	0	0	—	—	0	0	—	—	0	0	—	0	0

creased but at a rate considerably lower than that of inactivation of the infective property (Table III).

The Appearance of Non-Infectious Hemagglutinins and Complement-Fixing Antigens in HeLa Cells.—It has been shown by Schlesinger (3) that the neurotoxic effect of influenza virus in mice is accompanied by the appearance in the tissues of the CNS of complement-fixing antigen and of hemagglutinins in the absence of detectable increases in infectious virus, suggestive of an incomplete

cycle of viral reproduction. An experiment designed to test for the possible occurrence of incomplete reproductive cycles in HeLa cells is summarized below.

The medium was removed from culture bottles in which satisfactory sheets of cells had developed. The cells were washed thrice with Hanks's solution, and the sheets of 2 cultures each were then covered with 12 ml. amounts of (*a*) a 2-fold dilution of allantoic fluid infected with PR8 virus; (*b*) the same concentration of virus as that described under (*a*), but inactivated by ultraviolet light (3 minutes); and (*c*) active seed virus diluted to 10^{-2}. After 2 hours at 37°C. the virus suspensions were removed and the cells were washed 6 times with 10 ml. Hanks's solution each before the final maintenance medium was added. One bottle of each group was then harvested for base line determinations (2-½ hours after infection), the others were incubated at 36°C. From these 2 ml. aliquots were removed after 6 and 12 hours before

TABLE III

Cytopathogenic Effect of PR8 Virus Heated at 37°C. for Several Days

Seeds			Cytopathogenic effect (4th day of incubation)				
Period of heating at 37°C.	EID_{50}/ml.	HA/ml.	Dilution of seed				
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
days	*log*	*log*					
0	9.9	3.7	4	4	3	1	±
1	8.2	3.7	3	4	2	±	
2	7.2	3.7	1	2	±	0	
3	5.3	3.7	1	2	1	0	
4	4.8	3.7	1	2	0	0	

the final harvests were made in 24 hours. The cells of the bottles removed at 2-½ and 24 hours were scraped into the medium, the suspensions were then divided into 2 portions, one was kept native and the other was frozen and thawed 3 times in a dry ice–alcohol bath at −70°C. and a water bath at 37°C., respectively. The various preparations were then centrifuged at low speed and the supernates were assayed for infectivity and hemagglutinins.

The results of infectivity titrations and hemagglutinin assays are shown in Table IV. As can be seen, there were no significant increases in infectivity after 24 hours of incubation over the base line levels (2½ hours) representing residual seed virus. On the contrary, the titers tended to decrease slightly during the experimental period, presumably on account of inactivation at 37°C. On the other hand, the series employing active seed virus in dilution 1:2 showed definite evidence of hemagglutination in the medium by the 12th hour, when the cultures showed significant degrees of destruction, and the titer increased further to the 24th hour when the experiment was terminated. With the 100-fold diluted seed HA activity became barely detectable in 12 hours and the titer seen in 24 hours also was substantially lower than that obtained with seed in dilution 1:2. The extracts of the cells collected at the

end of the experiment likewise revealed significant HA activity, again roughly in proportion to the inoculum used. When ultraviolet-inactivated virus was employed no hemagglutinins were noted in either the medium or the cell extracts, and only a slight cytopathogenic effect was noted in 24 hours.

In further experiments the time of appearance of hemagglutinins as well as of the soluble (S) and the viral (V) antigens was determined and attempts

TABLE IV

The Appearance of Hemagglutinins in HeLa Cells Following Infection with PR8 Virus

Seed		Test	Hours of incubation			
Dilution	State		2½	6	12	24
$10^{-0.3}$	Active	Cytopathogenic effect	0	0	3	4
		EID_{50}/ml. (log), medium	6.6	6.0	6.3	5.9
		medium + cells*	6.3	—	—	6.6
		HA/ml. (log), medium	<0.4	<0.4	1.0	2.4
		medium + cells*	<0.4	—	—	2.4
10^{-2}	Active	Cytopathogenic effect	0	0	1	3
		EID_{50}/ml. (log), medium	4.6	4.3	4.0	3.8
		medium + cells*	4.8	—	—	4.3
		HA/ml. (log), medium	<0.4	<0.4	<0.4	1.2
		medium + cells*	<0.4	—	—	0.8
$10^{-0.3}$	3 min. U.V.	Cytopathogenic effect	0	0	0	1
		HA/ml. (log), medium	<0.4	<0.4	<0.4	<0.4
		medium + cells*	<0.4	—	—	<0.4

* The cells suspended in the medium were frozen and thawed 3 times and the supernate obtained after low speed centrifugation was used.

were made to study the activities in the cells and media separately. One of these is described below.

From each of 12 bottles of HeLa cells, showing good sheets, the medium was removed and replaced by 12 ml. of a 1:2 dilution of fresh allantoic fluid containing PR8 virus. 2 hours after inoculation the virus suspension was pipetted off, the cultures were washed 5 times with 10 ml. volumes of Hanks's solution and, after the last washing, 12 ml. of maintenance medium were added. The bottles were then returned to the incubator and one each was removed at 3, 4, 5, 6, 8, 12, 24, and 48 hours following infection. From the bottles collected up to the 12th hour, the medium was poured off for separate study and the cells were then scraped off the glass into fresh medium. After the 12th hour, separation of medium and cells was no longer possible in this experiment since in 24 hours many of the cells had sloughed off into the fluid

and, therefore, the remaining cells adherent to the glass were scraped into the medium before removal from the bottle. The various cell suspensions were used for analysis (*a*) as obtained; (*b*) after 1 cycle of freezing at −70°C. and thawing at 37°C. and additional sonic vibration for 15 minutes;[1] and (*c*) after centrifugation of preparation (*b*) at low speed to remove cellular debris. 1 ml. samples of all preparations were placed in ampuls and stored at −65°C. until EID_{50} titrations were possible. The remainder was used for hemagglutination and complement fixation tests. In addition, comparable preparations were collected at the 3, 24, and 48 hour intervals from uninfected cells for control purposes.

The results are presented in Fig. 1. The infectivity titers of the various separated cell suspensions obtained up to the 12th hour are represented by the solid symbols. As can be seen, the EID_{50} values in the disintegrated cell suspensions (*b*) and (*c*) were high 3 hours after infection and, therefore, denoted presumably residual seed virus which had not been removed by the washing procedures. The titers generally declined up to the 12th hour, indicating inactivation of the seed virus at 37°C. The untreated cell suspensions (*a*) with the exception of the 3rd hour sample revealed EID_{50} titers of the same order as those obtained after disintegration of the cells. The low value noted in the 3 hour sample may represent a chance observation or may denote that at this stage the seed virus is bound more firmly to the cells or cell constituents, so that it can be freed only by sonic vibration. Since after the 12th hour the medium no longer could be separated from the cells or cell debris, the EID_{50} values determined at 24 and 48 hours, indicated in the figure by the open symbols, represent the total concentrations of infectious virus in the cultures. These preparations, treated as the separated cell suspensions of the earlier harvests (*a*), (*b*), (*c*), revealed a loss in infectivity from the 1st to 2nd day of incubation (inactivation), but the 24 hour values were higher than those in the corresponding cell suspensions at 12 hours in the absence of medium. The increased titer in the 24 hour samples appeared to reflect the virus concentration in the medium rather than in the cells. Indeed, the medium separated at the 12 hour period contained more infectious virus than the cell suspensions obtained at that time. The difference recorded in the figure represents a minimum, since the endpoint had been underestimated in the planning of the titrations. The presence of considerable concentrations of residual seed virus in the medium poses an obstacle which has not been overcome.

This problem has not been apparent with respect to the hemagglutinating activity. Up to the 4th hour all tests were negative. Hemagglutinins became detectable in the untreated cell suspensions by the 8th hour and they increased thereafter steadily until the end of the experiment. However, when the cells were broken up by freezing and thawing and by sonic vibration, HA activity could be detected already in 5 hours; it increased rapidly up to 12 hours, and

[1] The sonic oscillator employed was manufactured by the Raytheon Manufacturing Company, Boston.

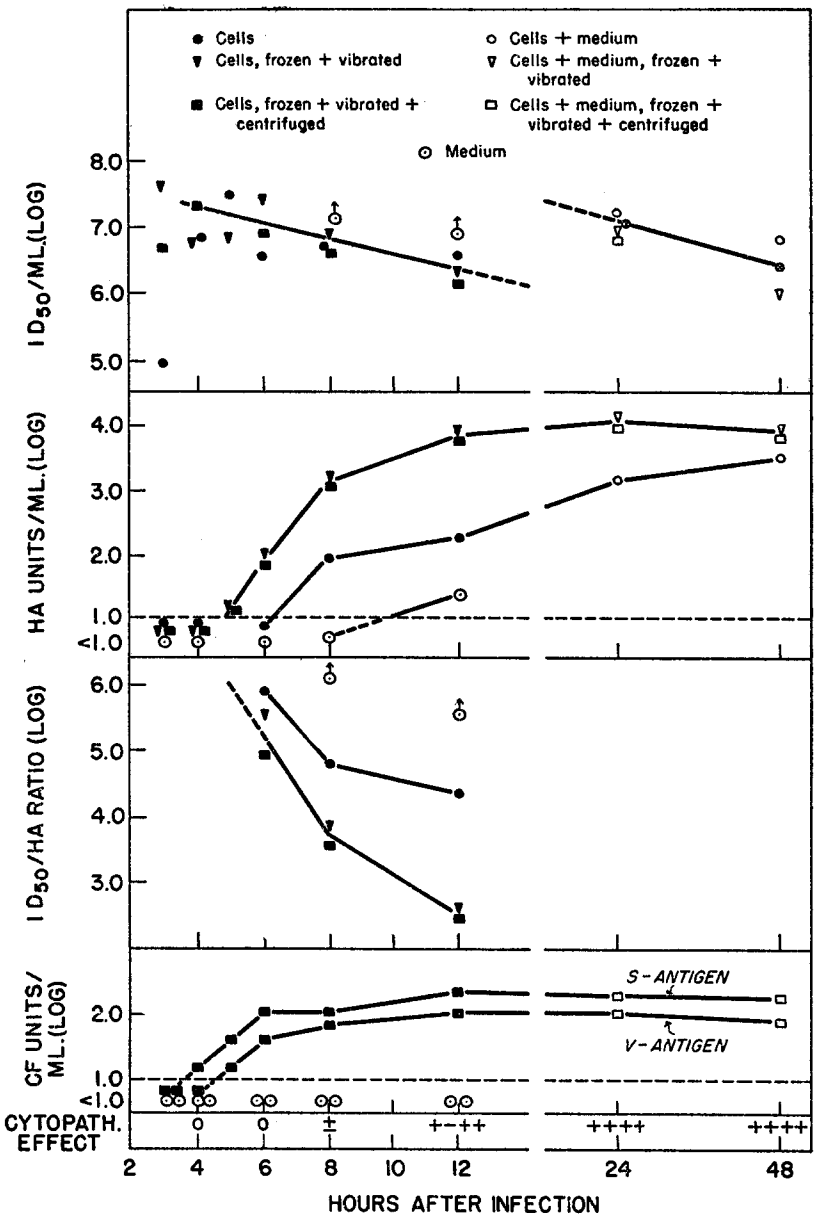

FIG. 1. The development of hemagglutinins and virus (V) and soluble (S) complement-fixing antigens in HeLa cells in the absence of detectable increases in infectious virus.

only slight increases were noted thereafter. Low speed centrifugation of the preparations did not alter the results. On the other hand, the medium collected separately during the first 12 hours revealed no HA activity up to the 8th hour and only a low titer in 12, when definite cytopathogenic effects became first apparent. These results indicated that the hemagglutinins are located largely within or on the cells and are not liberated into the medium unless the cells are destroyed in the course of incubation or by artificial disintegration.

As a result of the appearance of considerable hemagglutinin titers in the cells in the absence of a rise in infectivity the EID_{50}/HA ratios showed marked decreases from that of the seed virus in the early harvests ($>10^6$) to levels as low as $10^{2.5}$ in 12 hours in the disintegrated cell suspensions. The "intact" cell suspensions gave higher EID_{50}/HA ratios because only a fraction of the hemagglutinins could be detected under these conditions, whereas the infectivity titers of the variously treated preparations (*a*), (*b*), and (*c*) were closely similar. This apparent discrepancy may be explained by the fact that the HA titrations were performed on the fresh materials, whereas the samples for EID_{50} assays had been stored in the frozen state and suspension (*a*), therefore, no longer represented "intact" cells. The EID_{50}/HA ratios of the media were high and of the order of the seed virus employed.

Complement fixation tests were carried out with the disintegrated and centrifuged cell suspensions (*c*). The results showed that soluble (S) antigen could be detected by the 4th hour; *i.e.*, 1 hour before the appearance of both the virus antigen (V) and hemagglutinins. Maximal titers of the V and S antigens were reached in 12 hours and they remained at these levels for 48 hours without significant changes. The S titers throughout were somewhat higher than those of the V antigen. No complement-fixing activity was noted in the medium by the 12th hour.

Incorporation of P^{32} into the Hemagglutinating Component.—The experiments recorded above showed that hemagglutinins and complement-fixing antigens appeared in HeLa cells after incubation periods of 4 or more hours and increased thereafter to high levels without concomitant rises in infectivity. Furthermore, the height of the HA titers attained depended upon the dose of seed virus employed. It remained to be determined whether the hemagglutinins found represented the inoculated virus in some form or whether they had been produced in the infected cells. An answer to this question was sought by the use of an isotope (P^{32}). It has been observed previously that virus derived from the allantoic membrane of chick embryos injected with P^{32} prior to infection revealed considerable radioactivity, whereas virus exposed *in vitro* to the isotope failed to acquire this property (13, 7). Accordingly, attempts were made to determine whether radioactive hemagglutinins could be obtained from HeLa cells grown in the presence of P^{32}.[2]

[2] The advice and assistance of Dr. Oscar C. Liu in this experiment is gratefully acknowledged.

HeLa cells were grown in bottle cultures in the presence of P^{32} for two consecutive transfers according to the technic described in the section on methods and materials. The final cultures were then washed in the usual manner and to 12 bottles virus was added as in the above experiment (Series A). To another 12 cultures normal allantoic fluid in dilution 1:2 was added (Series B). A third series (C) consisted of 12 cultures grown under identical conditions in the absence of P^{32} and all of these were seeded with virus. After 2 hours of incubation the inocula were removed as described and to the maintenance media of Series A and B were added again 50 μc. of P^{32}. After 24 hours of incubation when the cells were largely destroyed the combined media and cells were collected, the mixtures were frozen and thawed once, subjected to sonic vibration for 15 minutes, and centrifuged at low speed. To Series C, P^{32} was now added in a concentration of 50 μc./12 cc. Finally, aliquots of the uninfected, labelled cell suspension obtained in Series B were mixed after disintegration with (*a*) standard virus (infected allantoic fluid) and incubated *in vitro* at 37°C. for 2 hours (Series D); (*b*) standard virus and incubated for 24 hours (Series E); and (*c*) an aliquot of the hemagglutinins obtained in Series C from P^{32}-free HeLa cells and incubated for 2 hours (Series F). These various suspensions (A-F) served as the starting materials for purification of virus and control materials, as described in detail elsewhere (7). Briefly, the materials were subjected to 2 cycles of adsorption onto and elution from red cells. The elutions into $\frac{1}{8}$ volume of saline solution were facilitated by addition of receptor-destroying enzyme of *Vibrio cholerae*[3] (RDE) to the cell suspensions in a concentration of 1 per cent. Following each elution the materials were dialyzed against 25 volumes of phosphate-buffered saline solution for 18 hours at 4°C. This technic was found to be satisfactory for almost complete removal of all free isotope and exclusion of labelled host materials. These various preparations were then assayed for radioactivity according to the method described elsewhere (7) and for hemagglutinating activity using 2 per cent sodium citrate–saline solution as the diluent. The remainder of preparations A, B, and C were divided into 2 equal portions each, chilled to 4°C. and absorbed at this temperature a third time with chicken red cells (1 per cent final concentration). After 60 minutes, the mixtures were centrifuged and the supernates were saved. The red cells obtained from one portion were washed in chilled saline solution and then digested in small amounts of fuming nitric acid. After the materials had cleared they were diluted in distilled water to the original volume for radioactivity assays. The sedimented red cells from the other portion were resuspended in the original volume of saline solution containing 1 per cent RDE and incubated at 37°C. for 90 minutes. After the elution period the red cells and media were separated by centrifugation and assayed as described above.

The results, presented in Table V, clearly show that only the hemagglutinins derived from infected radioactive cultures of HeLa cells (Series A) possessed radioactivity and that this activity was largely adsorbed onto and eluted from red cells concomitant with the HA activity. However, the adsorption of the hemagglutinin at 4°C. was somewhat more extensive than that of radioactivity indicating that the material possibly still contained labelled components other than hemagglutinins. The corresponding preparations of Series B, derived from uninfected cells grown in the presence of P^{32}, revealed no radioactivity above that of the background and, obviously, no HA activity. Thus, the purification procedure excluded labelled host components. The materials of Series C, likewise contained no detectable isotope activity, indicating that the hemagglutinins derived from normally grown HeLa cells were incapable of absorbing

[3] The preparation of RDE was generously supplied by Dr. Richard Haas of the Behring Werke, Marburg, Germany.

TABLE V

Incorporation of P^{32} into the Hemagglutinating Component Derived from HeLa Cells

Series	Starting material	Test material after 2 cycles of adsorption and elution	Assay		
			c.p.m./ml.	HA/ml.	c.p.m./HA
A	Infected P^{32} cell suspension (11.2×10^6 c.p.m./ml.)	Original	12,615	10,240	1.2
		60 min. 4°C., supernate	1,514	200	7.5
		RBC	7,575	—	—
		90 min. 37°C., supernate	7,790	7,680	1.1
		RBC			
B	Uninfected P^{32} cell suspension (12.2×10^6 c.p.m./ml.)	Original	0	<2.5	
		60 min. 4°C., supernate	0	<2.5	
		RBC	0	—	
		90 min. 37°C., supernate	0	<2.5	
		RBC	5	—	
C	Infected normal cell suspension P^{32} *in vitro* (13.2×10^6 c.p.m./ml.)	Original	0	7,680	
		60 min. 4°C., supernate	0	200	
		RBC	0	—	
		90 min. 37°C., supernate	0	3,840	
		RBC	0	—	
D	Uninfected P^{32} cell suspension + standard virus *in vitro*, 2 hrs. 37°C. (10.2×10^6 c.p.m./ml.)	Original	0	3,840	
D	Uninfected P^{32} cell suspension + standard virus *in vitro*, 24 hrs. 37°C. (10.2×10^6 c.p.m./ml.)	Original	0	3,840	
F	Uninfected P^{32} cell suspension + HA from normal cells *in vitro*, 2 hrs. 37°C. (10.2×10^6 c.p.m./ml.)	Original	0	1,920	

P^{32}. Finally, addition of standard virus in allantoic fluid or of the HA component derived from P^{32}-free HeLa cells to disintegrated, non-infected, radioactive cell suspensions failed to result in a combination of labelled host components with the two types of virus particles during incubation *in vitro* at 37°C. for up to 24 hours (Series D, E, and F). The results obtained support strongly the view that the hemagglutinins found in HeLa cells are produced therein.

DISCUSSION

The data presented show that influenza viruses may exert a cytopathogenic effect on HeLa cells, provided they are added to the cultures in large amount. No cellular destruction was observed with non-infected allantoic fluids or with preparations of virus which had been inactivated by prolonged exposure to ultraviolet light or 37°C. The effect was specifically neutralized by the addition of the appropriate immune sera to the culture media. The observations indicate that in the order of 10^7 EID_{50} of seed virus are required in order to destroy all the cells in culture tubes. With 1/10 to 1/100 the amount of seed only fractions of the cells die. Since the test tube cultures contained about $10^{5.3}$ cells at the time of inoculation it would seem that the cytopathogenic effect depends upon a high multiplicity of infection. However, it has not been possible as yet, on account of the inherent inaccuracies of the methods of assay, to determine in a reliable manner how much of the virus introduced into the cultures is actually adsorbed onto the cells. This problem requires further study.

The available evidence indicates that HeLa cells are incapable of supporting the production of fully infectious virus. (*a*) On serial passages of the agents in test tube cultures the cytopathogenic effect decreases consecutively in proportion to the dilution obtaining with the transfers of the standard virus seed employed for initiation of the series. Thus, by the 3rd or 4th passage cellular destruction became no longer apparent and little infectious virus could be detected by titration in chick embryos. (*b*) When limiting cytopathogenic doses of virus were used only a proportion of the cells were affected, even after prolonged incubation, indicating that the process did not spread from the cells initially infected by the seed to the non-infected ones. Finally, (*c*) infectivity assays in eggs showed that the titers in HeLa cultures were highest a few hours after infection and, thus, the infectious virus found represented residual seed virus, which had not been removed by the washing procedure. On further incubation of the cultures the EID_{50} titers decreased to some extent, indicating inactivation of the residual seed virus during incubation at 36°C. Although points (*a*) and (*b*) appear to exclude the occurrence of complete cycles of propagation, it would seem to be essential to achieve more extensive removal of the non-adsorbed seed virus following infection of the cells since the threshold level of infectivity established by it would prevent the detection of small quantities of infectious virus that might be produced.

In contrast to the infectivity data, considerable increases in hemagglutinin and of viral (V) and soluble (S) complement-fixing antigens became readily detectable in the infected cultures, but not when inactivated virus was used as seed (3 minutes U.V.). These determinations were not encumbered by residual seed levels of the respective activities. The HA titers ultimately attained seemed to be roughly proportional to the dose of seed virus employed. Hemagglutinins were found in the separated media only after the cytopathogenic effect had become apparent. On the other hand, they could be detected in extracts of the cells well in advance of cellular destruction. Furthermore, on physical disintegration of the remaining intact cells (or cellular debris), later in the incubation period, considerably larger quantities of HA became measurable than in the untreated cell suspensions. It is apparent therefore, that there exists no mechanism for release of the virus materials from the cells other than their disruption, be it the result of the cytopathogenic effect or of artificial means, such as freezing and thawing and sonic vibration.

These results resemble those obtained by Schlesinger upon intracerebral inoculation of mice with large doses of non-neurotropic strains of influenza virus (3). In that case, the quantities of non-infectious hemagglutinins found in brain extracts were larger by a factor of at least 10 over those injected and thus suggested the actual production of virus components in this organ. The experimental conditions in the present study were such as to cast doubt on the validity of corresponding calculations, which indicated at most that the yield equalled the input. For this reason, proof of the production of non-infectious hemagglutinins in HeLa cells—as against the suggestion of a return of adsorbed seed virus to a detectable form—was sought and found by isotope technics. The hemagglutinins derived from HeLa cells, which had been grown for 2 passages in the presence of P^{32}, revealed considerable radioactivity. In contrast, the HA component obtained from non-radioactive cultures or standard seed virus on exposure *in vitro* to soluble P^{32} or to a suspension of disintegrated labelled cells failed to acquire isotope activity. These results correspond to the experience gained with labelling of standard influenza virus in the chick embryo (13, 7). The data strongly suggest that hemagglutinins are formed in HeLa cells and that during this process P^{32} is incorporated into these components. Thus, the evidence supports the conclusion that HeLa cells are capable of supporting a partial or incomplete cycle of viral reproduction.

Such incomplete cycles do not seem to occur only in the CNS of mice (3) or in HeLa cells but evidence for the production of non-infectious hemagglutinins (NIHA) has been obtained also in the chorio-allantois of the chick embryo when the entodermal layer is infected with overwhelming doses of native (6, 14, 15) or partially heat-inactivated virus (16), or when the entodermal cells are treated with periodate prior to infection (17). Under the conditions cited, large quantities of virus materials are produced by the entodermal cells which

reveal EID_{50}/HA ratios of a strikingly lower order than those of standard virus preparations. It has not been determined at present whether the small quantities of infectious virus obtained are derived from a few cells producing only standard virus or whether all cells contribute to a greater or lesser extent. Thus, it is not possible to say to what extent the phenomena observed in the allantois and in HeLa cells are related. However, there are other similarities between the two systems which may be more than coincidental. Although HA and CF activities become detectable in the entodermal cells as early as 3 to 4 hours following infection whereas in HeLa cells, under the conditions employed, 1 to 2 hours later, this difference may reflect only the number of host cells available in these two cases. Since the HeLa cultures contained fewer cells than the entoderm (see text below), measurable quantities of HA or CF antigens are expected to be present in the latter at an earlier period. Furthermore, the quantities of HA produced per cell in the two systems do not appear to be strikingly different. After infection of chick embryos with large doses of seed virus the allantoic fluid collected 24 hours later, as a rule, contains between $10^{3.7}$ and $10^{4.1}$ HA units/ml., or, on the basis of an average volume of 8 ml. of fluid, a total of $10^{4.6}$ to $10^{4.9}$ units. These presumably are derived entirely from the entodermal cells the number of which has been estimated to be of the order of 10^8 (see reference 18). Thus, 0.0004 to 0.0008 HA units appear to be liberated per cell. HeLa bottle cultures contain on the average $10^{7.3}$ cells at the time of inoculation and these were found to yield totally between $10^{4.2}$ and $10^{4.8}$ HA units, or 0.0008 to 0.003 units per cell. The outstanding differences between HeLa and entodermal cells with respect to their interaction with influenza virus are the facts that the former are incapable of yielding infectious virus and that they release the virus material produced only upon disintegration, whereas from the latter infectious virus or non-infectious hemagglutinins are liberated in the absence of cellular destruction (19, 16). One might consider that the HeLa cells are destroyed so rapidly that completion of the virus is prevented. However, as has been pointed out, the HA and CF activities develop in both types of cells after comparable latent periods followed shortly thereafter in the entoderm by increases in infectivity; *i.e.*, by the 4th or 5th hour. Yet, significant degrees of cellular destruction in HeLa cultures are not seen before the 12th hour. It would seem, therefore, that the interval between first appearance of hemagglutinins and cytopathogenic effects is sufficiently long to offer a chance for completion of at least some infectious virus, if that were possible in this host cell. It is most likely that HeLa cultures are lacking in metabolites or enzyme systems which are required for reproduction of infectious virus.

Finally, it is of interest to note that the cytopathogenic effect was not restricted to native virus preparations. It was also produced, although to lesser extents, by virus partially inactivated by ultraviolet light or by heating at

37°C.; *i.e.*, with increasing exposure to the inactivating agents the rate of inactivation of the infectious property was considerably greater than that of cytopathogenicity. These results resemble those obtained with respect to the "toxic activities" of influenza virus (9, 12) which likewise are less susceptible to ultraviolet light or heat than infectivity. It has been suggested that the neurotoxic effect of influenza virus may be related to the incomplete reproductive cycle occurring in the CNS (3). In the present study no hemagglutinins were detectable in HeLa cells when exposed to ultraviolet-inactivated virus (3 minutes), although slight cytopathogenic effects were noted. This result does not necessarily contradict a relationship between "toxicity" and cytopathogenicity, on the one hand, and incomplete reproductive cycles, on the other, since it was found recently that partially heat-inactivated virus may produce considerable quantities of non-infectious hemagglutinins in the chick embryo (16), and that partially ultraviolet-inactivated virus is capable of inducing the phenomenon referred to as "multiplicity reactivation" (20). It is conceivable then that formation of hemagglutinins may have occurred in HeLa cells on exposure to ultraviolet-inactivated virus, but the quantities produced did not reach detectable levels.

SUMMARY

Various strains of influenza virus produce a cytopathogenic effect in cultures of HeLa cells. The virus could not be passed in series. Virus partially or even completely inactivated with respect to infectivity by exposure to 37°C. or ultraviolet light retained some of its cytopathogenic effect.

No evidence has been obtained of an increase in infectious virus in HeLa cultures, but an increase in hemagglutinins and in both viral and soluble complement-fixing antigens became detectable during incubation. These virus materials apparently were not released from these cells prior to their destruction.

These results suggested that HeLa cells are capable of supporting an incomplete reproductive cycle of influenza virus. The fact that radioactive phosphorus was readily incorporated into the hemagglutinin supplies strong evidence for this interpretation.

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