CORRELATION OF PRODUCTION OF INFECTIOUS VIRUS WITH SEQUENTIAL STAGES OF CYTOLOGIC ALTERATION IN HeLa CELLS INFECTED WITH ADENOVIRUSES TYPES 5 AND 7*

By GEORGIANNA S. BOYER, M.D., FLOYD W. DENNY, Jr., M.D., IRVING MILLER, and HAROLD S. GINSBERG, M.D.

(From the Departments of Preventive Medicine, Medicine and Pediatrics, School of Medicine, Western Reserve University, and the University Hospitals, Cleveland)

(Received for publication, July 8, 1960)

Adenovirus-infected tissue culture cells have been investigated by a variety of methods, including light and electron microscopy and cytochemical and fluorescent antibody techniques (1–7). The results of these studies have shown that adenoviruses produce characteristic sequential alterations in host-cell nuclei, and have demonstrated that certain of the structures which develop contain virus-like particles, deoxyribonucleic acid (DNA), and type-specific viral antigen. This evidence indicates that the intranuclear alterations described are manifestations of viral synthesis. The information provided by these studies is incomplete with respect to the role that sequential changes and various inclusions play in viral development because the techniques employed do not differentiate between infectious and non-infectious viral materials. Consequently, little is known of the infectivity of the particles and inclusions present in infected cells. Experiments were therefore designed to correlate the stages of adenovirus-induced cytologic change with production of infectious virus in HeLa cells. Adenoviruses types 5 and 7 were selected for investigation as being representative of two major subdivisions of the adenovirus group (7). The results of these studies form the basis for this report.

Materials and Methods

Viruses.—The prototype strains of the type 5 and 7 adenoviruses employed were furnished by Dr. R. J. Huebner.

Tissue Culture.—Details of the propagation of the HeLa cell line and the preparation, growth, and maintenance of the stationary tube cultures have been described elsewhere (8, 9).

* This investigation was conducted under the sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and was supported in part by the Office of The Surgeon General, Department of the Army, and by grants from the Brush Foundation, the Robert Hamilton Bishop, Jr. Endowment Fund, and the Republic Steel Corporation.

Experimental Design.—For correlative studies a large number of HeLa cell cultures were prepared in tubes, approximately one-half of which contained coverslips. The same media and techniques were used throughout for those cultures to be studied cytologically and those to be used to measure viral growth. The culture tubes were inoculated simultaneously with 0.1 ml. of viral suspension, and at the end of a 5 hour period to permit viral adsorption, the supernatant fluids containing unadsorbed virus were removed. Each culture was washed with 4 ml. of Hanks' balanced salt solution (BSS), after which 1 ml. of fresh maintenance mixture (8) was added. At selected intervals thereafter the supernatant fluids and cells from 4 replicate tube cultures were pooled and frozen for subsequent viral titration (9), and 4 to 6 companion coverslip cultures, including uninfected controls, were fixed and stained for microscopic study (6). The time intervals at which viral production and cellular changes were compared were selected on the basis of previous studies with the same viruses (7, 10). No attempt was made to follow the details of the cytologic process in the late, degenerative stages beyond 48 hours; *i.e.*, after the peak production of infectious virus had occurred.

The multiplication cycle of the virus was reconstructed from the results of infectivity titrations of the fluids and cells collected at each of the chosen intervals after infection. The pooled materials from each set of cultures were frozen and thawed 6 times, followed by centrifugation to remove cell debris. The virus-containing supernatant fluids were then stored frozen (−25° to −28°C.) until the infectivity titer of each pool was determined. Infectivity titrations were done using serial 1:3.2 ($10^{-0.5}$) dilution increments as previously described (9). The infectivity titer of each pool was defined as the highest dilution of virus in a 0.1 ml. inoculum which produced cytopathic changes in 50 per cent of the cultures 6 days after inoculation.

For cytologic studies the coverslip cultures were removed from culture tubes at selected intervals, fixed in 95 per cent ethyl alcohol, stored in 70 per cent ethyl alcohol, and later stained with hematoxylin and eosin. Prior to staining, the coverslip cultures were mixed and coded by an assistant so that at the time of microscopic observation the observer was unaware of either the interval after infection at which any given culture had been fixed or whether or not it had been infected. Three to 4 infected cultures and 2 control cultures were studied for each time interval; 100 cells were counted in each of 5 different areas on every coverslip culture examined. A minimum of 1500 cells was thus counted in infected cultures for each time interval. Each consecutive cell which appeared in the field of view was classified as to whether it appeared normal or infected and, if infected, to which stage of the cytologic sequence it belonged. The stages of the cytologic sequence were described in detail in earlier studies (7). The results of the differential counts were not assessed nor the coded slides identified until all cultures had been examined.

Statistical Methods.—Because it was not possible to determine experimentally the relative numbers of infectious units produced by cells in each of the various stages of infection, these were estimated statistically on the basis of certain assumptions. The following equation was used:

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4$$

Y is the relative number of infectious units at a given number of hours of infection; X_1 is the proportion of cells which were in stage 1 or had entered a subsequent stage by that time (*i.e.* those in stages 1, 2, 3, and 4); X_2, the proportion of cells in stage 2 or a later stage (those in stages 2, 3, and 4); X_3, the proportion of cells in stage 3 or 4; and X_4, the proportion of cells in stage 4. The b's, the quantities to be estimated, are the relative numbers of infectious units assumed to be produced in the respective stages. It was assumed in using this equation that the infectious units produced were stable and did not undergo inactivation during the cycle studied; previous studies indicated that adenoviruses were extremely stable under the experimental conditions employed (11). Finally, it was also assumed that there was no experimental error in estimating the percentage of cells in each stage.

The b values were estimated by a "least squares" method (14, 15). The nature of the experiment was such that the "error" in the infectivity titers was approximately constant. This implies that the "error" in the experimentally determined relative numbers of infectious units was a constant per cent. For example, an "error" of 10,000 at a titer of 1:100,000 had the same significance as an error of 100 at a titer of 1:1,000. This relationship was incorporated into the least squares solution by "weighting" each observation by the reciprocal of its Y value (*i.e.* dividing each of the 4 X's of an observation by its Y, and taking Y as "one").

EXPERIMENTAL

The evolution of characteristic virus-induced cytologic changes was compared with the production of infectious adenovirus in 3 separate experiments. The first 2 experiments were performed with type 5 adenovirus, and the third with type 7 virus. Detailed descriptions of the sequences of nuclear alterations which develop in HeLa cells infected with these 2 viruses were given in a previous paper (7) and therefore will not be repeated. The principal cytologic features of the various stages are, however, shown diagrammatically in Fig. 1.

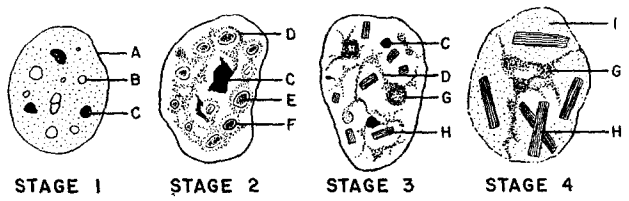
Type 5 Adenovirus

In the first study with type 5 adenovirus, cultures were inoculated with approximately $10^{5.15}$ TCD_{50} per 7×10^4 cells in an effort to infect a large proportion of the cells within a short time. Infected cultures were collected for study at a number of different intervals after infection; then, for each interval, the infectivity titer of the cells and supernatant fluid was measured in one set of infected cultures, and the per cent of apparently unaffected cells and the per cent of cells exhibiting changes characteristic of each of the 4 virus-induced cytologic stages (Fig. 1) were determined in a companion set. The results of the first experiment with type 5 adenovirus are given in Table I and Figs. 2 and 3.

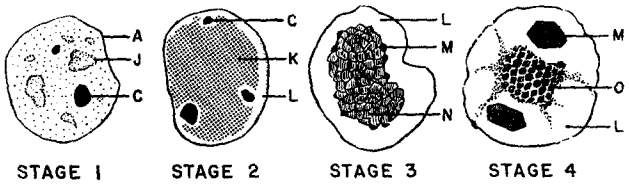
In Table I the per cent of cells which manifested characteristic virus-induced alterations (including all stages in the cytologic sequence) is compared with the amount of virus measured in companion cultures at each time interval studied. The amount of virus is expressed both in terms of infectivity titer and per cent of the maximum titer of virus produced during the multiplication cycle studied From the graphs in Fig. 2, in which the per cent of cells showing nuclear changes typical of viral infection (here not subdivided into stages) and the per cent of maximum viral titer reached at each time interval are plotted, it can be seen that the development of nuclear changes preceded the appearance of newly synthesized infectious virus. At 12 hours, 8 per cent of the cells already revealed well defined nuclear alterations but no new virus was detectable. The majority of infectious virus was not measured until several hours after characteristic virus-induced alterations had appeared in the majority of cells.

It was next of interest to relate the formation of infectious virus to the evolution of the individual stages of the cytologic sequence. Fig. 3 illustrates the per cent of cells in each of the 4 cytologic stages in cultures taken for study at the indicated intervals after infection. The sequential nature of the stages is

clearly shown by the maximum per cent of cells in stages 1, 2, 3, and 4 related to time after infection. Since the cytologic stages do occur in sequence, it may be assumed that each infected cell has already passed through the preceding stages. If developing viral particles should undergo maturation and become



TYPE 5



TYPE 7

FIG. 1. Diagrammatic representation of sequential stages of alteration which develop in nuclei of HeLa cells infected with the prototype strains of adenovirus types 5 and 7: *A*, nuclear membrane; *B*, eosinophilic inclusion; *C*, nucleolus; *D*, rearranged "chromatin"; *E*, granular basophilic core of inclusion; *F*, rim of inclusion; *G*, late stage basophilic, granular inclusion; *H*, eosinophilic; Feulgen-negative crystal-like structure; *I*, homogeneous, amphophilic background material; *J*, early irregular eosinophilic inclusion; *K*, central mass containing uniform granules; *L*, clear peripheral nuclear zone; *M*, basophilic, Feulgen-positive crystal; *N*, honeycomb-like central mass; *O*, central mass consisting of matrix and small crystals.

infectious during one particular stage in the sequence, then infectious virus would be expected to occur in cells in that and in subsequent stages.[1]

Fig. 3 also demonstrates the relative amount of infectious virus associated with cells in the 4 stages of infection. Stages 1 and 2 do not appear to be associated with infectious virus. At 16 hours, when over one-half of the cells revealed changes typical of stage 1, almost no infectious virus could be detected

[1] Previous studies have shown that adenoviruses are very stable and do not dissociate readily from infected HeLa cells; artificial cell disruption is necessary to induce release of the majority of infectious virus (9, 11).

(0.02 per cent), and 18 hours, when more than 40 per cent of the infected cells were in or had passed through stage 2, the per cent of virus which had been synthesized was still quite small (1.8 per cent). It was between 22 and 24 hours after infection that the curve representing the percentage of the maximum titer of infectious virus rose most steeply, when stages 3 and 4 were becoming predominant. Interpretation of the exact position and slope of the curve repre-

TABLE I

Comparison of Per Cent of Cells Showing Virus-Induced Changes with the Amount of Infectious Virus Measured in HeLa Cell Cultures Infected with $10^{5.25}$ TCD_{50} of Type 5 Adenovirus

No. of hrs. after infection	Per cent of cells showing characteristic virus-induced changes*					Amount of virus measured	
	Stage 1	Stage 2	Stage 3	Stages 4 and 5‡	Total all stages	Infectivity titer, TCD_{50}, log§	Per cent‖
8	1.0	0	0	0	1.0	−0.75¶	0.001
12	8.4	0	0	0	8.4	−0.75	0.001
14	27.3	0.85	0	0	28.2	—	—
16	58.8	11.2	0	0	70.0	−2.0	0.02
17	56.0	14.6	0.2	0	70.8	−3.0	0.18
18	43.9	37.3	3.8	0	85.0	−4.0	1.8
19	38.4	34.0	10.8	0.1	83.3	−4.25	3.0
20	26.0	37.3	24.7	4.3	92.3	−4.75	10.0
22	18.0	30.8	36.3	7.5	92.6	−5.0	17.8
24	12.2	23.6	38.8	21.5	96.1	−5.75	100.0
30	4.2	9.6	28.7	54.4	96.9	−5.75	100.0

* For each interval a total of 2000 cells was counted on 4 different coverslip cultures; the percentage figures given are averages of the findings.

‡ Cells in stage 5, rare before 30 to 48 hours, were included in stage 4 when they were encountered.

§ Infectivity titer = highest dilution of virus in 0.1 ml. inoculum which produced cytopathic effects within 6 days in 50 per cent of the cultures inoculated.

‖ Per cent of maximum titer reached in experiments.

¶ The virus measured at 8 and 12 hours represents virus from the original inoculum remaining despite the washing procedure, not newly synthesized virus.

senting infectious virus and its relation to the other 4 curves describing the progression of the cytologic stages will not be attempted because of the limitations in quantitating infectious adenovirus and in accurately performing cell counts.

The above evidence suggests that HeLa cells showing characteristic early changes of infection with type 5 adenovirus contain only small amounts of infectious virus. In order to demonstrate this observation in a more objective manner, the relative numbers of infectious units associated with each of the stages of infection were exhibited by the "least squares" solution as de-

scribed in Materials and Methods (Table II). Using estimated b's hypothetical infectivity titers were determined for each time period studied during the multiplication cycle and these titers were compared to those determined experimentally. This comparison appears in Fig. 4. It is apparent that determined and computed infectivity titers were very similar. The specific b values suggest that almost no infectious particles were associated with stages 1 and 2,

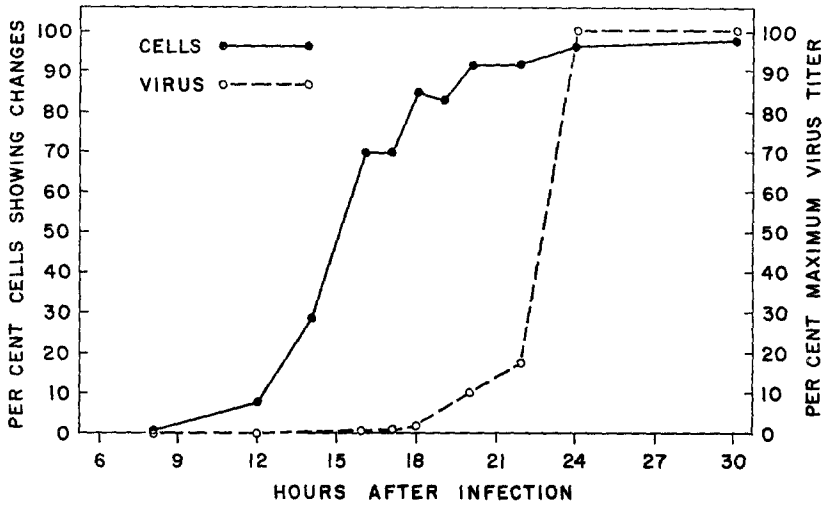


FIG. 2. Comparison of the per cent of cells showing cytologic changes characteristic of infection with the production of infectious virus in HeLa cell cultures infected with $10^{5.25}$ TCD_{50} of type 5 adenovirus. A large number of tissue culture tubes, half of which contained coverslips on which cells were growing, were simultaneously inoculated with virus. At each indicated time period, 4 tubes without coverslips were harvested, their contents pooled and after all specimens were collected, infectivity titrations were performed. At the same time periods, coverslips were removed from companion tubes, fixed, stained with hematoxylin and eosin, and examined for changes due to type 5 infection.

and that over 3 times as many infectious units were associated with stage 4 as with stage 3.

The second experiment with adenovirus type 5 was carried out with a smaller infecting inoculum ($10^{4.0}ID_{50}$/HeLa cell culture of approximately 7×10^4 cells) to determine whether the relationships between the cytologic stages and production of infectious virus observed in the first experiment could be confirmed or whether they were merely coincidental occurrences under the experimental conditions employed.

The results of the second experiment are presented in Table III and Figs. 5 and 6. It is apparent that the data obtained in the second experiment are quantitatively but not qualitatively different from those observed in the first, in which a larger viral inoculum was used. First, considering the cytologic

findings, it is evident that within the experimental period employed fewer cells became infected when exposed to the smaller inoculum: 30 hours after infection only 20 per cent of the cells revealed morphologic signs of infection, as compared to 97 per cent in the first experiment. The stages of nuclear change progressed more slowly (compare Figs. 3 and 6). The qualitative nature of the

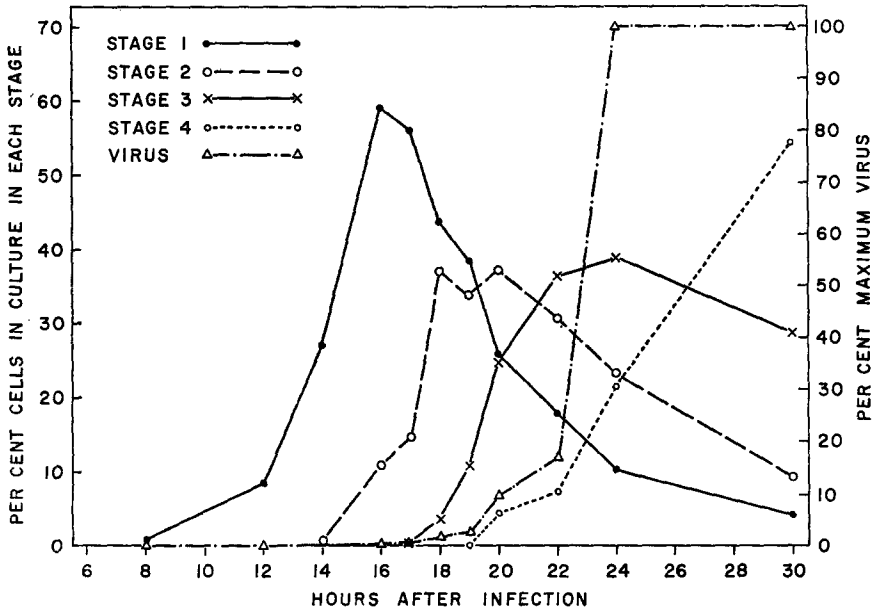


FIG. 3. Comparison of the virus infectivity titer with the progression of stages of sequential cytologic change in HeLa cells infected with $10^{5.25}$ TCD_{50} of type 5 adenovirus. Tissue culture tubes, half of which contained coverslips on which cells were growing, were simultaneously inoculated with virus. Three or 4 coverslips were removed at each indicated time and stained with hematoxylin and eosin; a minimum of 1500 cells was examined at each time period and the per cent of cells in each of the 4 stages of infection was determined. At each time period 4 tubes without coverslips were harvested, their contents pooled, and infectivity titrations performed on all specimens simultaneously.

cytologic alterations did not differ, however, in the two experiments. In regard to the production of infectious virus, the maximum viral titer reached in the second experiment was lower and was not attained until later after inoculation (48 hours as compared to 24). Most significant, however, was the finding that the development of infectious virus showed fundamentally the same relationship to the progression of the cellular changes that was observed in the first experiment. The appearance of cytologic changes preceded the detection of newly formed infectious virus (Fig. 5); cells in stage 1 appeared to lack infectious virus, and the largest amounts of virus per cell were present in association

with the late stages of cytologic change, particularly stage 4 (Fig. 6). Table IV and Fig. 7 demonstrate the marked degree of similarity between the infectivity titers determined in the experiment described and the infectivity titers computed by the "least squares" method. As in the experiment using the larger amount of type 5 virus, the b values in this experiment suggest that very little

TABLE II

Comparison of Observed Infectivity Titers with Infectivity Titers Computed by the "Least Squares" Method in HeLa Cell Cultures Infected with $10^{5.25}$ TCD_{50} of Type 5 Adenovirus (Derived from Data in Table I)

No. of hrs. after infection	Proportion of cells in 4 stages of cytological change*				Y‡			
					Observed		Computed with b's	
	X_1	X_2	X_3	X_4	No.	Log§	No.	Log§
8	1.0	0	0.0	0.0	1	0	0.13	0‖
12	8.4	0	0.0	0.0	1	0	1.10	0
14	28.2	0.85	0.0	0.0	No observation	—	4.40	0.75
16	70.0	11.20	0.0	0.0	18	1.25	19.00	1.25
17	70.0	14.80	0.2	0.0	178	2.25	82.00	2.00
18	85.0	41.10	3.8	0.0	1,780	3.25	1,200.00	3.00
19	83.3	44.90	10.9	0.1	3,170	3.50	3,400.00	3.50
20	92.3	66.30	29.0	4.3	10,000	4.00	13,000.00	4.00
22	92.6	74.60	43.8	7.5	17,800	4.25	21,000.00	4.25
24	96.1	83.90	60.3	21.5	100,000	5.00	40,000.00	4.50
30	96.9	92.70	83.1	54.4	100,000	5.00	79,000.00	5.00
b¶	0.13	0.90	300.0	1,000.0				

* X_1 is the proportion of cells in stages 1 to 4 at indicated time; X_2 is the proportion in stages 2 to 4; X_3, those in stage 3 or 4; X_4, those in stage 4.

‡ Y is the relative number of infectious units at the indicated time.

§ The logarithms were taken to the nearest 0.25.

‖ The logarithm was taken arbitrarily as zero.

¶ b is the relative number of infectious units estimated to be produced upon entrance into each stage.

infectious virus was associated with cytopathic stages 1 and 2 and that large quantities of infectious virus were associated with stages 3 and 4.

Thus, the result of the second experiment, employing an inoculum containing $10^{4.0}ID_{50}$ of type 5 adenovirus, confirmed the relationship between the cytologic alterations and the production of infectious virus found in the first experiment, in which approximately 18 times more virus was used as an infecting inoculum.

Adenovirus Type 7

Type 7 adenovirus was found to be less suitable for quantitative cytologic study than type 5 because the differences between the stages of cellular alteration produced by type 7 were less distinct (7). The results of only one experiment correlating the virus multiplication cycle and the cytologic changes evoked by type 7 virus will be presented.

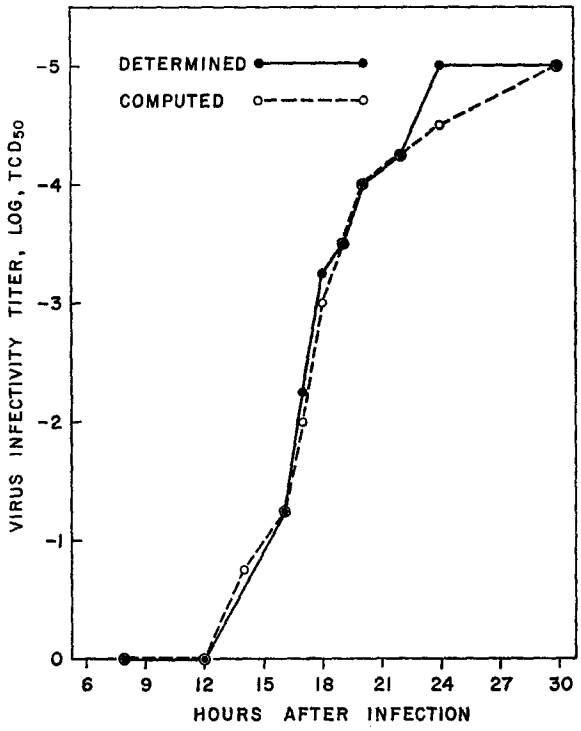


FIG. 4. Comparison of infectivity titers determined after infection of HeLa cells with $10^{5.25}$ TCD_{50} of type 5 adenovirus with titers which were computed from a fitted equation (see text).

An inoculum containing $10^{3.25}$ TCD_{50} of type 7 virus was used and a 5 hour period allowed for viral adsorption. The results of this experiment are illustrated in Table V, and in Figs. 8 and 9. Table V and Fig. 8 reveal that typical cytologic alterations (shown diagrammatically in Fig. 1) were apparent before any newly synthesized virus was detected, and that the development of the initial cytologic changes in the majority of cells preceded the appearance of the majority of the mature infectious virus. Fig. 9 demonstrates that the stages of cytological change occurred sequentially, as was the case with type 5 virus; *i.e.*, that

TABLE III

Comparison of Per Cent of Cells Showing Virus-Induced Changes with the Amounts of Virus Measured in HeLa Cell Cultures Infected with $10^{4.0}$ TCD_{50} of Adenovirus Type 5

No. of hrs. after infection	Per cent of cells showing characteristic virus-induced changes*					Amount of virus measured	
	Stage 1	Stage 2	Stage 3	Stages 4 and 5	Total all stages	Infectivity titer TCD_{50}, log	Per cent‡
6	0	0	0	0	0	<0	0
14	1.1	0	0	0	1.1	<0	0
16	1.7	0	0	0	1.7	<0	0
18	2.7	0.2	0	0	2.9	<0	0
20	4.9	0.5	0	0	5.4	−0.50	0.03
22	7.0	1.0	0.3	0	8.3	−1.00	0.10
24	6.4	2.7	0.6	0	9.7	−2.00	1.00
30	7.8	5.1	5.2	0.9	19.0	−3.25	17.80
40	2.9	2.8	7.8	8.0	21.5	−3.75	56.20
48	1.2	1.0	4.1	14.4	20.7	−4.00	100.00

* For each interval a total of 1500 cells was counted on 3 different coverslip cultures; the percentage figures given are averages of the findings.

‡ Per cent of maximum titer reached in experiments.

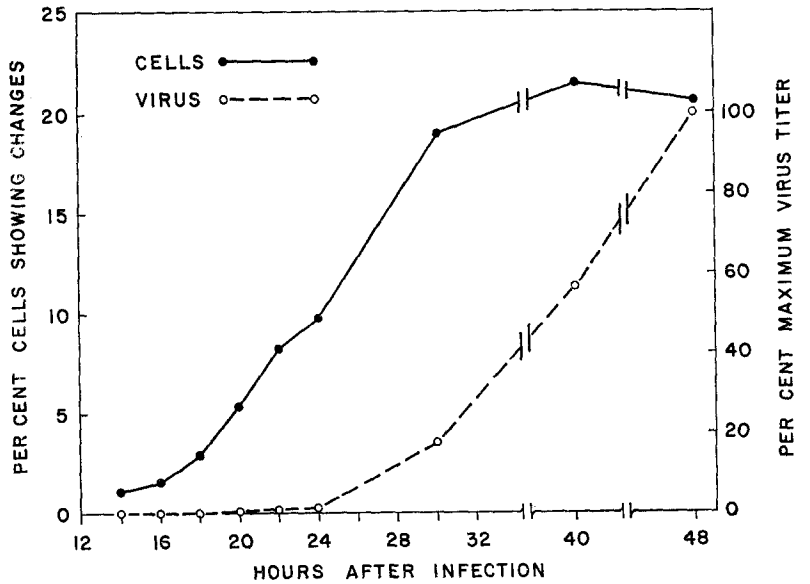


FIG. 5. Comparison of the per cent of cells showing cytologic changes characteristic of infection with the production of infectious virus in HeLa cell cultures infected with $10^{4.0}$ TCD_{50} of type 5 adenovirus. The experiments were done exactly as described in legend for Fig. 2.

adenovirus type 7-infected cells in stages 1 and 2 were associated with little mature infectious virus, and that cells in stages 3 and 4 were associated with large amounts.

A comparison of the observed infectivity titers with the titers calculated by the "least squares" method is shown in Table VI and Fig. 10. It can be seen that the observed and calculated values are remarkably similar. The *b* values (estimated relative quantities of infectious virus) were very low for stages 1 and 2 (0.15 and 0.17, respectively) and much higher for stages 3 and 4 (84 and 390,

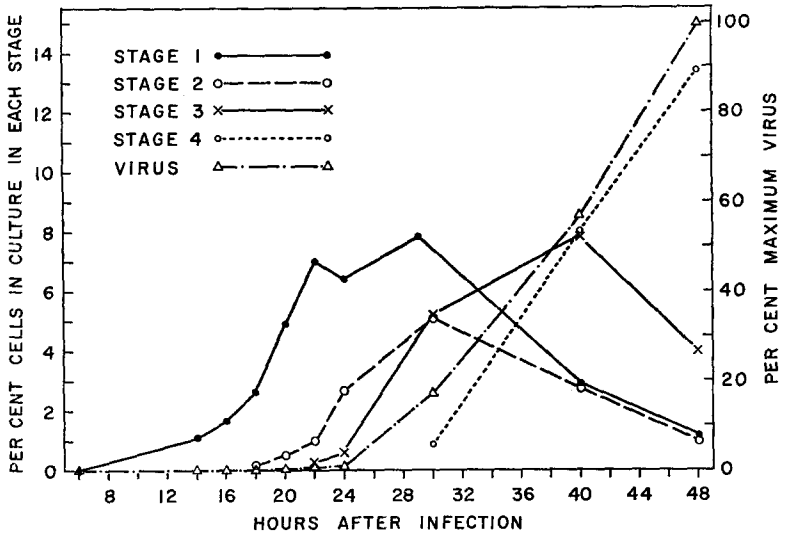


FIG. 6. Comparison of the virus infectivity titer with progression of stages of sequential cytologic change in HeLa cells infected with $10^{4.0}$ TCD_{50} of type 5 adenovirus. This experiment was done exactly as described in legend for Fig. 3.

respectively). Thus, the experiments with type 7 adenovirus are in close agreement with the experiments with the type 5 virus and suggest that with the two adenovirus types studied the majority of infectious virus was associated with cells in the late stages of infection.

DISCUSSION

The results of the studies reported above correlating the development of adenovirus-induced cytological changes with the production of infectious virus confirm and extend previous observations which indicated that the intranuclear alterations were manifestations of viral synthesis (1–7). In experiments with both adenovirus type 5 and type 7, nuclear changes preceded the appearance of newly synthesized infectious virus. The relationship between the development of nuclear alterations and the production of virus remained the same, although

the proportion of cells which manifested virus-induced changes, the rate at which the changes progressed, the maximum titer of virus produced, and the length of time required for the maximum virus titer to be attained, varied with the size of the inoculum.

The evidence obtained is consistent with the hypothesis that cells in the

TABLE IV

Comparison of Observed Infectivity Titers with Infectivity Titers Computed by the "Least Squares" Method in HeLa Cell Cultures Infected with $10^{4.0}$ TCD_{50} of Type 5 Adenovirus (Derived from Table III)

No. of hrs. after infection	Proportion of cells in 4 stages of cytological change*				Y‡			
					Observed		Computed with b's§	
	X_1	X_2	X_3	X_4	No.	Log‖	No.	Log‖
6	0	0	0	0	1	0	0	0¶
14	1.10	0	0	0	1	0	0.66	0¶
16	1.70	0	0	0	1	0	1.00	0
18	2.90	0.2	0	0	1	0	1.70	0.25
20	5.40	0.5	0	0	3	0.50	3.20	0.50
22	8.30	1.3	0.3	0	10	1.00	15.00	1.25
24	9.70	3.3	0.6	0	100	2.00	26.00	1.50
30	19.00	11.2	6.1	0.9	1780	3.25	850.00	3.00
40	21.50	18.6	15.8	8.0	5620	3.75	6200.00	3.75
48	20.70	19.5	18.5	14.4	10,000	4.00	11,000.00	4.00
b**	0.60	−2.7	34.0	700.0				

* X_1 is the proportion of cells in stages 1 to 4 at indicated time; X_2 is the proportion in stages 2 to 4; X_3, those in stage 3 or 4; X_4, those in stage 4

‡ Y is the relative number of infectious units at the indicated time.

§ In these computations b_2 (−2.7) was taken arbitrarily as zero.

‖ The logarithms were taken to the nearest 0.25.

¶ These logarithms were taken arbitrarily as zero.

** b is the relative number of infectious units estimated to be produced upon entrance into each stage.

first and second stages of nuclear alteration, although actively engaged in viral synthesis, contain little or no mature infectious virus, whereas cells in the later stages contain relatively large amounts. This hypothesis is in accord with other data concerning the nature of the cell structures which characterize the various stages, as discussed below.

In the case of infection with adenovirus type 5, the inclusions present during stage 1 are eosinophilic and Feulgen-negative, and thus appear to lack, or contain very little DNA (7). Fluorescent antibody studies reveal that stage 1 cells generally contain less viral antigen than do cells in the succeeding stages (7). Unfortunately, it is not feasible to correlate the light microscopy and

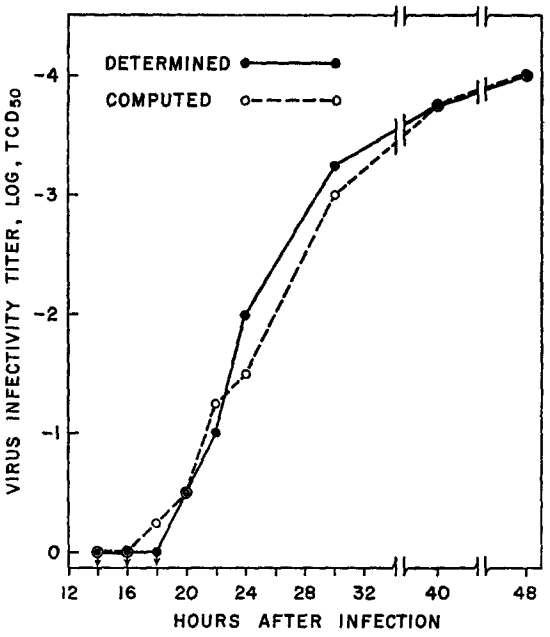


FIG. 7. Comparison of infectivity titers determined after infection of HeLa cells with $10^{4.0}$ TCD_{50} of type 5 adenovirus with titers which were computed from a fitted equation (see text).

TABLE V

Comparison of Per Cent of Cells Showing Virus-Induced Changes with the Amounts of Infectious Virus Measured in HeLa Cell Cultures Infected with $10^{3.25}$ TCD_{50} of Adenovirus Type 7

No. of hrs. after infection	Per cent of cells showing characteristic virus-induced changes*					Amount of virus measured	
	Stage 1	Stage 2	Stage 3	Stage 4	Total all stages	Infectivity titer TCD_{50}, log	Per cent‡
6	0	0	0	0	0	−0.25	0
10	0.4	0	0	0	0.4	—	—
12	2.0	0	0	0	2.0	0	0
14	7.0	0.3	0	0	7.3	0	0
15	—	—	—	—	—	0	0
16	11.5	1.6	0	0	13.1	−0.50	0.02
17	—	—	—	—	—	−1.50	0.18
18	16.2	4.4	0.4	0	21.0	−1.75	0.32
19	—	—	—	—	—	−2.00	0.56
20	20.7	8.2	2.5	0.3	31.7	−2.50	1.80
22	16.2	11.0	10.5	5.5	43.2	−3.50	17.80
24	17.3	17.4	9.6	4.2	48.5	−4.00	56.00
30	8.0	6.6	19.4	24.4	58.4	−4.25	100.00
40	—	—	—	—	—	−4.50	>100.00
48	5.9	3.4	11.0	35.8	56.1	−4.25	100.00

* For each interval studied a minimum of 1500 cells was counted on 3 different coverslip cultures; the percentage figures given are averages of the findings.

‡ Per cent of maximum titer reached in experiments.

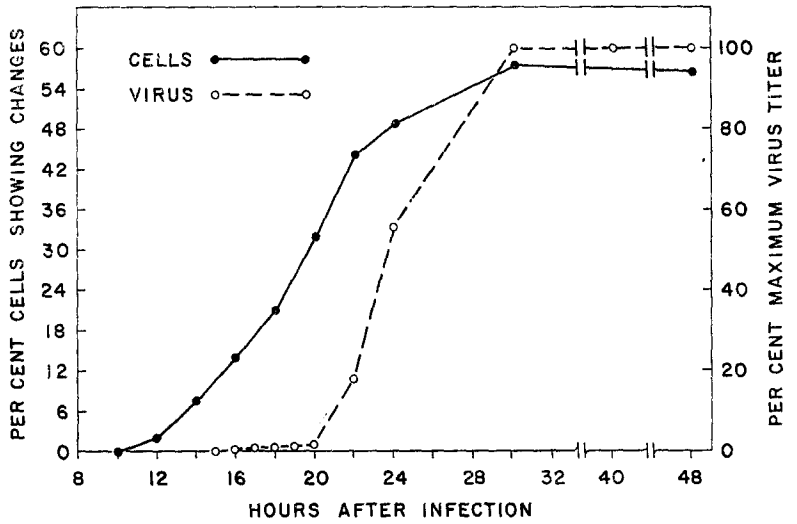


FIG. 8. Comparison of the per cent of cells showing cytologic changes characteristic of infection with the production of infectious virus in HeLa cell cultures infected with $10^{3.25}$ TCD_{50} of type 7 adenovirus. The experiments were done exactly as described in legend for Fig. 2.

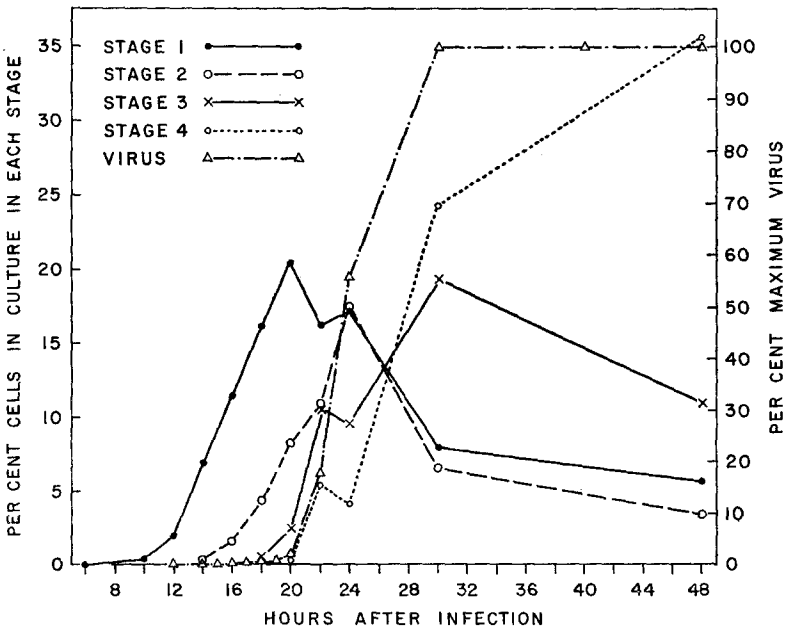


FIG. 9. Comparison of the virus infectivity titer with progression of stages of sequential cytologic change in HeLa cells infected with $10^{3.25}$ TCD_{50} of type 7 adenovirus. The experiments were done exactly as described in legend for Fig. 3.

TABLE VI

Comparison of Observed Infectivity Titers with Infectivity Titers Computed by the "Least Squares" Method in HeLa Cell Cultures Infected with $10^{3.25}$ TCD_{50} of Type 7 Adenovirus (Derived from Data in Table V)

No. of hrs. after infection	Proportion of cells in 4 stages of cytological change*				Y‡			
					Observed		Computed with b's	
	X_1	X_2	X_3	X_4	No.	Log§	No.	Log§
6	0	0	0	0	1.0‖	0	0	0¶
10	0.4	0	0	0	No observation	—	0.06	0¶
12	2.0	0	0	0	1.0	0	0.30	0¶
14	7.3	0.3	0	0	1.0	0	1.10	0.00
15	No observation				1.0	0	—	—
16	13.1	1.6	0	0	3.2	0.50	2.20	0.25
17	No observation				32.0	1.50	—	—
18	21.0	4.8	0.4	0	56.0	1.75	38.00	1.50
19	No observation				100.0	2.00	—	—
20	31.7	11.0	2.8	0.3	320.0	2.50	360.00	2.50
22	43.2	27.0	16.0	5.5	3,200.0	3.50	3500.00	3.50
24	48.5	31.2	13.8	4.2	10,000.0	4.00	2800.00	3.50
30	58.4	50.4	43.8	24.4	18,000.0	4.25	13,000.00	4.00
40	No observation				18,000.0**	4.25	—	—
48	56.1	50.2	46.8	35.8	18,000.0	4.25	18,000.00	4.25
b‡‡	0.15	0.17	84.0	390.0				

* X_1 is the proportion of cells in stages 1 to 4 at indicated time (*i.e.* those in stages 1, 2, 3, and 4); X_2 is the proportion in stages 2 to 4; X_3, those in stage 3 or 4; X_4, those in stage 4.

‡ Y is the relative number of infectious units at the indicated time.

§ The logarithms were taken to the nearest 0.25.

‖ Table V shows −0.25 for the infectivity titer, but this was taken arbitrarily as zero titer.

¶ These logarithms were taken arbitrarily as zero.

** Table V shows −4.5 for this infectivity titer, but this was taken arbitrarily as −4.25 titer.

‡‡ b is the relative number of infectious units estimated to be produced upon entrance into each stage.

fluorescent antibody observations of the early stages of type 5 infections reported (7) with the electromicroscopic findings reported by other investigators (12, 13). It is thus impossible to state whether the cells here designated as stages 1 and 2 contain virus-like particles or not. It would be of interest to determine whether cells corresponding to these stages contain precursor matrix material or recognizable viral particles, possibly in an early stage of development. Those cells which are associated with infectious virus (stages 3 and 4 and possibly 2) possess inclusions which contain DNA as well as specific viral antigen, in contrast to cells in stage 1. From the electron micrographs of Morgan

et al. (13), it is evident that regular arrays of well developed virus-like particles are present in type 5 adenovirus-infected cells in stages 3 and 4; cells in these stages can be identified by the presence of the eosinophilic, bar-shaped type of crystal.

In regard to type 7 adenovirus infection, the inclusions of cells in the first stage of the cytologic sequence lack both DNA and type-specific viral antigen, although some viral antigen which cross-reacts with type 5 antiserum is already present (7). These stage 1 inclusions probably correspond to the "reticulated"

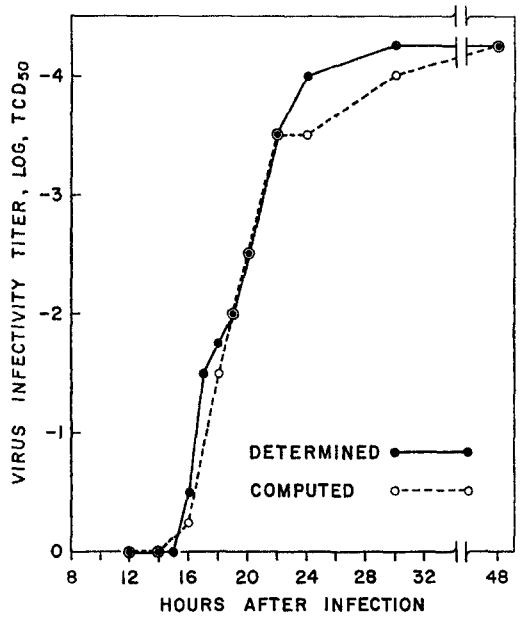


FIG. 10. Comparison of infectivity titers determined after infection with $10^{8.25}$ TCD_{50} of type 7 adenovirus, with the titers which were computed from a fitted equation (see text).

condensations of dense granular "matrix" observed by electromicroscopists in nuclei of HeLa cells considered to be in early stages of infection with adenovirus types 3, 4, and 7 (3). The crystals characteristic of infection with type 7 virus (and also types 3 and 4), are first apparent by light, phase, and fluorescence microscopy in the second stage of cytologic change, and become increasingly prominent in stages 3 and 4; crystals of this sort have been shown to contain DNA, specific viral antigen, and virus-like particles (3, 4, 6, 7). The correlative studies described above indicate that infectious virus is produced by cells which contain such crystals; *i.e.*, cells in stages 3 and 4, and possibly 2.

One other finding of interest in the present studies was the high percentage of HeLa cells that could be infected nearly simultaneously under the conditions

employed. This observation is in contrast to data reported by other investigators (2), which indicated that only a small percentage of cells in HeLa cultures became infected when exposed to adenoviruses. This discrepancy in the proportion of cells which appear infected presumably is related to the size of the infecting inoculum and the susceptibility of the cells under different experimental conditions.

SUMMARY

Studies correlating the production of infectious adenovirus (types 5 and 7) and the progression of the stages of virus-induced cytologic change in HeLa cells are presented. The results reveal a close relationship between the development of the characteristic nuclear changes and adenovirus synthesis. They suggest that cells manifesting the first stages of nuclear change, characterized by the appearance of eosinophilic, Feulgen-negative inclusions, contain little or no mature infectious virus, whereas cells in the later stages, with Feulgen-positive and basophilic inclusions, contain relatively large amounts.

The authors are grateful to Dr. George F. Badger and Dr. Arthur S. Littell for advice with the statistical analyses of the data.

BIBLIOGRAPHY

1. Kjellen, L., Lagermalm, G., Svedmyr, A. M., and Thorsson, K. G., Crystalline-like patterns in the nuclei of cells infected with an animal virus, *Nature*, 1955, **175**, 505.
2. Harford, C. G., Hamlin, A., Parker, E., and van Ravenswaay, T., Electron microscopy of HeLa cells infected with adenoviruses, *J. Exp. Med.*, 1956, **104**, 443.
3. Morgan, C., Howe, C., Rose, H. M., and Moore, D. H., Structure and development of viruses observed in the electron microscope. IV. Viruses of the RI-APC group, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 351.
4. Block, D. P., Morgan, C., Godman, G. C., Howe, C., and Rose, H. M., A correlated histochemical and electron microscopic study of the intranuclear crystalline aggregates of adenovirus (RI-APC virus) in HeLa cells, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 1.
5. Barski, G., Caractère spécifique de la lésion cellulaire causée *in vitro* par les virus du groupe A.P.C. et sa valeur diagnostique, *Ann. Inst. Pasteur*, 1956, **91**, 614.
6. Boyer, G. S., Leuchtenberger, C., and Ginsberg, H. S., Cytological and cytochemical studies of HeLa cells infected with adenoviruses, *J. Exp. Med.*, 1957, **105**, 195.
7. Boyer, G. S., Denny, F. W., Jr., and Ginsberg, H. S., Sequential cellular changes produced by types 5 and 7 adenoviruses in HeLa cells and in human amnion cells, *J. Exp. Med.*, 1959, **110**, 827.
8. Ginsberg, H. S., Gold, E., and Jordan, W. S., Jr., Tryptose phosphate broth as a supplementary factor for maintenance of HeLa cell tissue cultures, *Proc. Soc. Exp. Biol. and Med.*, 1955, **89**, 66.

9. Ginsberg, H. S., Characteristics of the adenoviruses. III. Reproductive cycles of types 1 to 4, *J. Exp. Med.*, 1958, **107,** 133.
10. Denny, F. W., Jr., Boyer, G. S., and Ginsberg, H. S., Characteristics of adenovirus types 5, 6, and 7, *Fed. Proc.*, 1958, **17,** 509 (abstract).
11. Ginsberg, H. S., Characteristics of the new respiratory viruses (adenoviruses). II. Stability to temperature and pH alterations, *Proc. Soc. Exp. Biol. and Med.*, 1956, **93,** 48.
12. Lagermalm, G., Kjellen, L., Thorsson, K. G., and Svedmyr, A., Electron microscopy of HeLa cells infected with agents of the adenovirus (APC-RI-ARD) group, *Archiv. Ges. Virusforsch.*, 1957, **7,** 221.
13. Morgan, C., Godman, G. C., Rose, H. M., Howe, C., and Huang, J. S., Electron microscopic and histochemical studies of an unusual crystalline protein occurring in cells infected by type 5 adenovirus. Preliminary observations, *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 505.
14. Snedecor, G. W., Statistical Methods, Ames, Iowa State College Press, 5th edition, 1956, chapter 14.
15. Deming, W. E., Statistical Adjustment of Data, New York, John Wiley & Sons, Inc., 1943.