CHARACTERIZATION OF A FACTOR FORMED IN THE COURSE OF ADENOVIRUS INFECTION OF TISSUE CULTURES CAUSING DETACHMENT OF CELLS FROM GLASS

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Although adenovirus infection of HeLa cells is characterized by slow adsorption of virus (1, 2) and a latent period of 14 to 21 hours (2–4), the effects of low dilutions of infectious tissue culture material can be detected within a few hours after inoculation. Boyer *et al.* (5) reported cytopathic effects within "several hours," and we have occasionally observed cytopathic effects 4 to 6 hours after inoculation of low dilutions of adenovirus suspensions. Also, Levy *et al.* (6) reported stimulation of radioisotope uptake by adenovirus infected HeLa cells beginning within 2 to 4 hours after inoculation. During the course of the latter study, another early alteration was observed, namely, that the infected cultures tended to detach from the glass wall of the culture vessel when rinsed within several hours after inoculation; the same observation has been noted by Ginsberg (2). By subjecting inoculated HeLa cell cultures to a controlled degree of agitation on a shaking machine, a reproducible system for demonstrating the decrease in cell adherence was devised. This report describes the characteristics of the cell detachment phenomenon, and presents evidence that it is produced by a virus-specific factor distinct from the infectious virus particle.

Materials and Methods

Tissue Cultures.—Stationary tube cultures of HeLa cells were obtained from Microbiological Associates, Inc., Bethesda. The cultures were prepared by seeding with 81,000 trypsin-dispersed cells in 0.6 ml. of a medium consisting of 20 per cent pooled human serum in Eagle's basal medium (BME)[1] (7). They were received in this laboratory when 2 days old, at which time they were rinsed 3 times with 1.0 ml. of BME, and given 1.0 ml. of a maintenance medium

[1] BME, Eagle's basal medium.

containing 5 per cent chicken serum, 25 per cent tryptose phosphate broth, and 70 per cent medium 199 (8); this modification of the Ginsberg medium (9) was recommended to us by Mr. Julius A. Kasel of the Laboratory of Clinical Investigation of the National Institutes of Health. In most cell detachment experiments the cultures were used on the following day. All tissue culture media contained penicillin and streptomycin, in concentrations of 100 to 250 U and 100 to 250 μg., respectively.

Viruses.—The prototype adenovirus strains (10) were used. Adenoviruses of human and chimpanzee origin were grown in 32 ounce flask cultures of HeLa or KB cells; the fluids and cells were harvested 2 days after complete cytopathic effects, and frozen at −20°C. The suspension was thawed, clarified by centrifugation at 2500 R.P.M. for 20 minutes, and the supernatant fluid was dispensed into vials which were stored at −20°C. *Rhesus* kidney cultures were used for preparation of pools of adenoviruses of monkey origin, poliovirus, and Coxsackie B3 virus. Herpes virus, vaccinia virus, and the Mills strain of hemadsorption virus type 1 (11, 12) were grown in tube cultures of HeLa cells. Control inocula were prepared by harvesting fluid and cells from uninfected flask cultures after 5 to 7 days of incubation, and treating in the same manner as described for the virus pools.

Tests for Cell-Detaching Activity.—In the standard test, 0.1 ml. of test material was added to the medium present in the tube; generally 2 tubes were inoculated with each dilution of test material, but occasionally 3 to 5 tubes were used. Each test included three sets of controls: 2 to 4 uninoculated cultures; 2 to 4 cultures inoculated with 0.1 ml. of control tissue culture fluid–cell material, at a dilution equal to the lowest dilution of virus in the test; and a titration of a standard pool of type 2 adenovirus. The cultures were incubated at 36°C. for 4 hours, at which time they were read microscopically. The tubes, in spring racks[2] at a 5° slant, were then placed on a reciprocating shaking machine[3] with a frequency of 270 to 300 oscillations per minute and an amplitude of $1\frac{9}{16}$ inches. The direction of shaking was perpendicular to the length of the tube. The tubes were shaken for 2, for 10, and then for 15 minutes; after each period of shaking the extent of loss of cell sheet was determined by gross inspection. A tube was considered positive if more than half the cell sheet had detached after the full 27 minutes of shaking. In the infrequent instances when control tubes lost appreciable amounts of tissue the experiment was discarded.

Infectivity and Cell Detachment Titrations.—Infectivity titrations were performed by inoculation of 0.1 ml. of serial 10-fold dilutions into each of two HeLa cell tube cultures. The cultures were observed for 12 to 14 days, depending on the condition of the tissue, and the fluids were changed twice weekly. A tube was considered positive if three-fourths or more of the cells demonstrated cytopathic effects; titers are expressed as the reciprocal of the dilution, (calculated by the Reed-Muench method (13)) which would produce CPE[4] in 50 per cent of tubes in the time interval stated in the text, when inoculated with 0.1 ml. volume (ID_{50} per 0.1 ml.). When comparisons of infectivity titers were made, all titrations were done simultaneously in the same lot of cultures.

Titrations of cell-detaching activity were carried out by inoculating 0.1 ml. of serial 1:3.16 (half-log) dilutions into each of two HeLa cell cultures. Dilutions were made in the regular HeLa maintenance medium. The cell detachment test was performed and graded as described above; reproducibility between replicate tubes was generally very good. Titers are expressed as the reciprocal of the dilution (calculated by the Reed-Muench method (13)) which would produce cell detaching effect in 50 per cent of the tubes when inoculated with 0.1 ml. of test material (CDU_{50}[5] per 0.1 ml.).

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[2] Drummond Scientific Co., Philadelphia, Pennsylvania.

[3] Arthur H. Thomas Co., Cat. No. 8917A.

[4] CPE, cytopathic effects.

[5] CDU_{50}, cell detaching $units_{50}$.

Serological Tests.—Complement fixation tests were performed as described elsewhere (14); for determination of CF antibody titers, crude tissue culture fluid antigens were used. Antigen titrations were made by testing against pooled convalescent serum of persons infected with several different adenovirus types.

Tests for adenovirus neutralizing antibody were made in monkey kidney tissue cultures, as described previously (9, 14). Antibody titers are expressed in terms of the dilution of serum before addition of virus and culture medium, which is 10-fold lower than the final dilution of serum in the culture tube.

Inhibition of cell detaching activity was determined in a similar manner. A dilution of infectious tissue culture fluid was prepared to contain 1.8 to 5.6 CDU_{50} per 0.1 ml.; generally this was a 1:3.2 dilution of the infectious tissue culture fluid. One volume of virus dilution, one volume of serum dilution, and 8 volumes of HeLa maintenance medium were mixed, allowed to stand at room temperature for approximately 15 minutes, and 1.0 ml. was transferred to each of two HeLa cell cultures from which the medium had been removed. Controls included one tube receiving serum dilution, and a cell detachment titration. The usual 4 hour incubation and shaking were carried out. A serum dilution was considered positive for cell detachment inhibition if both tubes showed less than 50 per cent cell detachment; the great majority of serums giving positive results completely prevented cell detachment. Only tests in which the simultaneous titration of cell detaching activity yielded results corresponding with titers established in previous titrations are included in the results.

EXPERIMENTAL

Characteristics of the Cell Detachment Effect

General Description.—Four hours after inoculation with low dilutions of type 1 or 2 adenovirus, HeLa cell sheets generally showed slight cytopathic effects, consisting of retraction and rounding of cells at the upper portion or sides of the cell sheet. Marked cytopathic effects occasionally occurred, consisting of generalized separation, rounding, and clumping of cells, without the granulation characteristic of late stages of adenovirus reproduction in the cells. After shaking, the cell sheet demonstrated cell loss ranging from ragged or diffuse thinning of the sheet, seen with light cultures, or stripping of patches of cell sheet, seen generally in heavier cultures, to complete loss of cells from the wall of the tube. Cell detachment by type 2 adenovirus was produced in HeLa cells, KB cells (15), and monkey heart (16) cultures, all of which are continuous passage cell lines, but not in primary trypsinized cultures of human amnion or monkey kidney.

Batches of HeLa cells varied in sensitivity to the cell detaching effect; the titer of a standard suspension of adenovirus type 2 varied from 6 to 180 CDU_{50} per 0.1 ml., but in the majority of tests was 18 to 56 CDU_{50} per 0.1 ml. With cultures from the same lot of cells, titers were relatively constant in cells tested under different conditions, but the degree of cytopathic effects and duration of shaking needed to produce detachment varied markedly. An experiment illustrating this variation is recorded in Table I; a suspension of type 2 adenovirus was titrated four times in the same lot of HeLa cells in cultures which had been held in growth and maintenance medium for different periods.

TABLE I

Cell Detachment by Adenovirus Type 2 in HeLa Cells of Different Ages

Age of cells		Inoculum		Results after 4 hr. incubation at 36°C.			
Days in growth medium	Days in maintenance medium	Material	Dilution	CPE	Cell loss after shaking for indicated times*		
					2 min.	12 min.	27 min.
		None		−, −	0; 0	0; 0	0; 0
2	0	Control, TC fluid	1:3.2	−, −	0; 0	0; 0	0; 0
		Type 2 adenovirus	1:3.2	±, ±	±, ±	3; 3	4; 4
			1:10	−, −	2; ±	3; ±	4; 2
			1:32	−, −	0; 0	±; 1	±, 1
			1:100	−, −	0; 0	0; 0	0; 0
		None		−, −	0; 0	0; 0	0; 0
2	1	Control, TC fluid	1:3.2	−, −	0; 0	0; 0	0; 0
		Type 2 adenovirus	1:3.2	±, ±	0; 0	4; 4	
			1:10	−, −	0; 0	3; ±	4; 3
			1:32	−, −	0; 0	0; 0	0; 0
			1:100	−, −	0; 0	0; 0	0; 0
		None		−, −	0; 0	±; 0	1; 0
3	0	Control, TC fluid	1:3.2	−, −	0; 0	0; 0	0; 0
		Type 2 adenovirus	1:3.2	+++, +++	4; 4		
			1:10	+, ++	2; 3	4; 4	
			1:32	−, −	0; 0	0; 0	0; 0
			1:100	−, −	0; 0	0; 0	0; 0
		None		−, −	0; 0	0; 0	0; 0
4	1	Type 2 adenovirus	1:3.2	++, ++	4; 4		
			1:10	±, ±	2; 1	4; 3	4; 4
			1:32	−, −	0; 0	1; 1	2; 2
			1:100	−, −	0; 0	0; 0	0; 0

* Numerals indicate extent of detachment of cell sheet in duplicate tubes. 0, no detachment, 4, complete detachment.

The cell-detaching titer was relatively constant, but the degree of cytopathic effects and rapidity of detachment differed greatly. In this experiment the rapidity of detachment tended to parallel cytopathogenicity, but this parallel was not a consistent finding in other experiments. It has been observed repeatedly that older cells freshly changed to maintenance medium demonstrated the most marked cytopathic effects.

Composition of the maintenance medium did not appear to influence the test; variations in medium composition tested included BME with 5 per cent chicken serum; BME with 5 per cent inactivated (56°C. for 30 minutes) horse serum; regular HeLa maintenance medium plus 1, 5, or 10 per cent unheated

TABLE II

Dilutions of Type 2 Adenovirus Suspension Producing Cytopathic Effects and Cell Detachment in HeLa Cell Cultures after Different Periods of Incubation

Length of incubation at 36°C.	Titer by CPE		Titer by cell detachment (CDU_{50}).
	Minimal CPE ($\geq \pm$)	Marked CPE ($\geq ++$)	
hrs.			
0	<1	<1	<1
½	<1	<1	1.8
1	<1	<1	3.2
2	1.8	<1	5.6
4	5.6	<1	56
8	32	<1	56
24	560	18	56

calf serum; regular medium plus 10 per cent unheated ox serum (two lots of serum tested); or regular medium plus 5 per cent unheated guinea pig serum.

Incorporation of Ca^{++} and Mg^{++} ions into the regular maintenance medium in double (2.5 and 3.9 mM, respectively) the concentration present in medium 199 did not affect the cell-detaching titer. Also, preincubation of virus suspension for 72 hours at 4°C. with an equal volume of medium containing Ca^{++} and Mg^{++} ions at concentrations of 25.2 and 39.0 mM, respectively, did not affect cell-detaching titer.

The temperature of incubation affected the degree of cell detachment activity. Infectious type 2 tissue culture fluid was titrated in three replicate sets of HeLa cell cultures, which were then held for 4 hours at 4, 20, and 36°C. The cell detachment titers after shaking were 1, 1, and 18 CDU_{50} per 0.1 ml, respectively.

Rapidity of Action of Cell Detachment Factor.—Table II presents results of a representative experiment to determine the effect of incubation for various intervals of time on the cell detachment and cytopathic effect titers of type 2 test material. HeLa cell culture tubes were inoculated with 0.1 ml. of serial

half-log dilutions of virus, and after various periods of incubation at 36°C., two cultures inoculated with each dilution as well as four uninoculated cultures and four cultures inoculated with 0.1 ml. of control KB cell culture fluid, were examined microscopically and subjected to the shaking procedure. Shaking immediately after inoculation did not produce detachment, but detachment was produced by the undiluted material within 30 minutes after inoculation. The dilution producing detachment rose progressively through the 4th hour, and remained unchanged at 8 and 24 hours. Cytopathic effects were first seen at 2 hours, but were minimal in extent; severe effects did not occur until after 8 hours of incubation. As noted above, cytopathic effects did not parallel cell detachment.

Failure to Demonstrate Uptake of Cell Detachment Factor by HeLa Cells.—To test for disappearance of cell-detaching factor following reaction with HeLa cells, two types of experiment were done. Ten HeLa cell tube cultures, each containing 1 ml. regular maintenance medium, were inoculated with 0.1 ml. of type 2 adenovirus suspension. After 4 hours incubation at 36°C., the cultures were subjected to the usual shaking procedure. The contents of the tubes were pooled and centrifuged at 1500 R.P.M. for 15 minutes; the supernatant fluid was collected and stored at 4°C. The following day the medium was removed from 9 HeLa cell cultures, and each was inoculated with 1 ml. of the supernate; these cultures were then incubated and shaken by the standard procedure, and the contents pooled and centrifuged as before. This procedure was repeated until the original suspension had been exposed to 5 sets of HeLa cultures; at each of the 5 passages the cultures showed detachment following shaking. When the test fluid and a control dilution prepared in cell-free maintenance medium and held at the same temperatures as the test virus were titrated simultaneously in a sixth set of cultures, they yielded identical titers of 1.8 CDU_{50} per 0.1 ml.

In the second experiment, 30 HeLa cell cultures were inoculated with 0.1 ml. of infectious type 2 tissue culture fluid, and placed at 36°C.; at each of the intervals shown below 5 cultures were removed from the warm room and the culture fluids removed, pooled, and placed immediately at −50°C. Cell-detaching titers, determined in a single test, were as follows: 0 time, 18; 15 minutes, 18; 30 minutes, 32; 1 hour, 18; 2 hours, 18; and 4 hours, 18 CDU_{50} per 0.1 ml., respectively. Although these titrations are subject to a probable 3-fold error, it is evident that the bulk of the detaching factor was neither inactivated nor lost through irreversible binding to HeLa cells. The evidence also indicates that the cell-detaching factor is not being formed in detactable quantities during the first 4 hours following exposure of the cells to infectious tissue culture fluid.

Viruses Producing Cell Detachment.—Many adenovirus types, as well as a number of tissue culture fluids from cells infected with other agents were tested for ability to produce cell detachment in HeLa cell cultures. Infectious tissue culture fluids of adenovirus types 1 through 9, 11, 14, 16, C-1, and M-3 pro-

duced detachment, but types 3, 4, 7, and M-3 were generally low in activity. Adenovirus types 10, 12, 13, 15, all of which reproduce relatively poorly in HeLa and KB cells, did not produce cell detachment, nor did poliomyelitis type 1 (Mahoney strain), Coxsackie B3, herpes simplex, vaccinia, or a newly recognized myxovirus (Mills strain of HA virus type 1), when tested in the standard 4 hour test. The cell-detaching activity of the adenovirus types was not correlated with CF antigen titers of the preparations tested.

Allantoic fluid infected with the PR8 strain of influenza A, which hemagglutinated 0.5 per cent chick red blood cells to a titer of 1:320, did not produce cell detachment.

TABLE III

Growth Curve of Cell-Detaching Activity, Infectivity, and Complement-Fixing Antigen in HeLa Cell Cultures Inoculated with 10^{-3} Dilution of Adenovirus Type 2 (Approximately $10^{4.5}$ TC ID_{50})

Time of harvest after inoculation	CPE on day of harvest	CF antigen titer	Infectivity titer (14 days)	Cell detachment titer
days				*CDU_{50} per 0.1 ml.*
3	−	<8	$10^{3.5}$	<1
4	±	<8	$10^{5.0}$	<1
5	++	32	$10^{6.5}$	5.6
6	++++	256	$10^{7.5}$	18
7	Tissue gone	128	$10^{7.0}$	18
8	" "	128	$10^{6.0}$	18
9	" "	160*	$10^{6.5}$	32

* Titer determined in different test.

Time of Appearance of Cell-Detaching Factor in Infected HeLa Cell Cultures.— Six HeLa cell culture tubes were inoculated with 0.1 ml. of a 10^{-3} dilution of type 2 adenovirus, and the culture fluid was changed 24 hours later. On days 3 to 6, 0.3 ml. of culture fluid was removed from each tube and replaced with fresh medium; on days 7 to 9, the entire contents of two tubes were harvested. The pooled fluids from each day were titrated for CF antigen, infectivity, and cell-detaching activity (Table III). Cell-detaching activity was first detected on the 5th day after inoculation, at which time definite cytopathic effects first appeared; on the following day the cell detachment titer had risen, and cytopathic effects were complete.

Properties of the Factor Producing Cell Detachment

Studies of the chemical and physical nature of the factor-producing cell detachment were carried out with type 2 adenovirus infectious tissue culture fluids. A number of active preparations were dialyzed against balanced salt solution or tap water; in all instances, full activity was recovered in the non-

dialyzable fraction. Full activity was also recovered in the precipitate formed by half saturation with ammonium sulfate; ammonium sulfate precipitation followed by dialysis of the resuspended sediment provided a useful method for obtaining 5- or 10-fold concentration of cell-detaching activity. Acid–45 per cent methanol precipitation, which is useful for concentration of adenovirus complement fixing antigen (17), was not effective in concentrating cell-detaching activity. The fractions in Table I of the report by Forsyth and Roizman (17) were tested for cell-detaching activity; the starting material, with CF antigen titer of 260, had a CDU_{50} titer of 18; fraction 5 had a CF titer of 1700 and a detachment titer of 56; fraction 8 a CF titer of 63,000 and detachment titer of 56; while fraction 9, with CF antigen titer of 30,000, had a detachment titer of less than 10. Thus, the two activities did not concentrate in parallel; detaching activity was not recovered quantitatively in any of the fractions.

Overnight exposure of infectious tissue culture fluid to 20 per cent diethyl ether did not affect the cell-detaching titer.

Effect of Heat.—Boyer *et al.* (5) reported that the ability of adenovirus suspensions to produce early cytopathic effects was not abolished by heating at 56°C. for 5 minutes. Similarly, cell-detaching activity was not affected by heating at 56°C. for 5 or 10 minutes, but a small amount of infectivity remained; heating for 30 minutes at 56 or 60°C., which completely inactivated virus infectivity, reduced, but did not destroy cell-detaching activity. Heating at 70°C. for 30 minutes completely destroyed cell-detaching activity.

Effect of Ultraviolet Irradiation.—Tests for sensitivity to ultraviolet light were kindly conducted by Drs. Samuel Baron and Abraham Miller. Ten ml. of type 2 virus suspension was placed in a 90 mm. diameter Petri dish on a rotary shaker below a 15 watt General Electric germicidal lamp; radiation intensity was 36 microwatts per sq. cm. as measured with an Archer radiation meter. The untreated virus preparation had an infectivity titer of $10^{6.5}$ and a cell-detaching titer of 3.2, while suspension irradiated for 3 hours had an infectivity titer of $10^{2.5}$, with a cell-detaching titer of 5.6. Thus, cell-detaching factor was much more resistant to ultraviolet irradiation than viral infectivity.

When heated or ultraviolet-irradiated preparations were tested for infectivity, it was generally observed that at 24 or 48 hours after inoculation of 0.1 ml. of undiluted or 10^{-1} dilution of the sample, the HeLa cell cultures demonstrated 2+ or 3+ cytopathic changes; the same observation has been described by Pereira and Kelly (18). Within several days the changes regressed, and the cultures appeared normal; but if the preparation still contained viable virus, typical viral cytopathic effects subsequently appeared, and the cultures were completely destroyed. This indicated that the toxic effect of the inactivated virus did not produce irreversible damage to the cells, and did not interfere with their ability to support the growth of virus.

Effects of Enzymes.—In order to gain insight into the chemical nature of the

material responsible for the cell-detaching effect, active preparations were treated with trypsin, ribonuclease, and desoxyribonuclease. Crystalline ribonuclease and desoxyribonuclease (Nutritional Biochemical Co.) and crystalline trypsin (Armour) were used; solutions of 1 mg./ml. were prepared in 0.02 M phosphate buffer, pH 7.4. The desoxyribonuclease solution contained in addition 0.03 M $MgCl_2$. Two preparations of adenovirus type 2 were used; No. 1 was prepared by infecting HeLa cells with 1 volume of undiluted virus per 9 parts serum-free maintenance medium, harvesting the cells at 24 hours, and disrupting them in a French cell press;[6] the solution was dialyzed against

TABLE IV

Cell-Detaching Activity and Complement-Fixing Antigen Titer of Type 2 Adenovirus after Treatment with Enzymes

Material	Test	Enzyme treatment			
		None	RNAase	DNAase	Trypsin
No. 1. Concentrated serum-free virus	Cell detachment (1:3.2)	+	+	+	−
	CF antigen titer	1:128	1:128	1:128	1:128
Concentrated control fluid	Cell detachment (1:3.2)	−	−	−	−
No. 2. Standard virus	Cell detachment (1:3.2)	+	+	+	−
	CF antigen titer	1:64	1:64	1:64	1:64
Standard control preparation	Cell detachment (1:3.2)	−	−	−	−

water, and lyophilized; the dried material was resuspended in pH 7.4 phosphate buffer. A control preparation was prepared from uninfected HeLa cell cultures in the same manner. Preparation 2 was a standard KB pool of type 2 at pH 7.4; the standard uninfected KB cell–fluid material was used as control. One volume of enzyme was added to 15 volumes of virus and control materials, and the mixtures, as well as the materials without added enzyme, were incubated in closed vials for 3 hours at 37°C.; they were then held at 4°C. for 6 days until tested. Simultaneously with the enzyme treatment, the enzyme preparations were tested for activity against yeast RNA, calf thymus DNA, and crystalline bovine serum albumin; each enzyme was active and specific for its appropriate substrate. Assay for cell-detaching activity was made by testing each mixture at a dilution of 1:3.2; the results are shown in Table IV.

The trypsin-treated virus preparations showed no activity in the cell detachment test, while the preparations treated with ribonuclease and desoxyribonu-

[6] Pressure cell; American Instrument Company, Silver Spring, Maryland.

clease showed activity identical with the untreated materials. The uninfected preparations produced no cell detachment, indicating that the enzymes alone did not produce detachment under the conditions used. In contrast to cell-detaching activity, complement-fixing antigen titer was not affected by any of the enzyme preparations.

Centrifugation Studies.—Because of the marked dissociation of cell detaching activity from infectivity in heated or ultraviolet-irradiated preparations, it was desirable to determine if the two activities were produced by different physical particles. Fractionation experiments were done by the ultracentrifugation of infectious tissue culture fluid in a sucrose density gradient, since this provided a suitable method of separating particles of different size or density without inactivating infectivity.

Sucrose density gradient tubes were prepared by successively layering 0.5 ml. each of 56, 51, 45, 37, 29, 21, and 11 per cent *w/w* sucrose solutions prepared in isotonic saline; the tubes were held at 4°C. overnight, and 0.5 ml. virus suspension was placed on top the column. The tubes were then centrifuged in a Spinco No. SW39L swinging bucket rotor at 4°C. in Spinco model L ultracentrifuge, at 30,000 R.P.M. (71,000 *g* average) for 2 hours. Successive layers of fluid were removed by needle aspiration through the wall of the celluloid tube; the layers were labelled alphabetically, the uppermost layer being "sucrose fraction A," etc. When the same virus material was centrifuged in two or three tubes, the corresponding layers were removed in essentially equal amounts and were pooled. In some experiments the pellet was resuspended in the lowest layer of the supernatant fluid, and in other cases was resuspended in 0.5 or 0.8 ml. of saline per tube. The effect of sucrose on the infectivity of virus contained in infectious tissue culture fluid was studied in Experiment 3. At the time of the fractionation of the virus in sucrose density gradient, an aliquot of the infectious tissue culture fluid undergoing ultracentrifugation was diluted 1:4 in 11, 37, and 56 per cent *w/w* sucrose solutions prepared in isotonic saline. The starting materials for the three experiments were as follows: No. 1, the 9th day harvest of HeLa cell tube cultures infected with 0.1 ml. of 10^{-3} dilution of virus; No. 2, extracellular virus harvested 48 hours after inoculation of KB cells with one volume undiluted virus per nine parts serum-free maintenance medium; and No. 3, a standard pool of type 2 virus, prepared as described in Methods. The results of assays of the fractions for virus, complement-fixing antigen, and cell-detaching activity are shown in Table V.

In the first experiment the bulk of the cell detaching factor as well as the soluble CF antigen was found in fractions A and B. Although only about 1 per cent of the infectivity of the starting material was recovered, over 95 per cent of the recovered virus was found in the bottom fraction (D and pellet). The loss of virus was probably due to poor extraction of an almost invisible pellet. Considerably better recovery of infectious virus was effected in Ex-

periments 2 and 3. In all three experiments, as shown in Table V, (columns 5, 7 and 9), the bulk of the infectious virus localized in the lower fractions while the

TABLE V

Separation of Cell-Detaching Factor from Infectious Virus Contained in Infectious Tissue Culture Fluid by Ultracentrifugation in a Sucrose Density Gradient

Experiment No.	Material	Volume	CDU_{50} per 0.1 ml.	CDU_{50} total	ID_{50} (12 days) per 0.1 ml.	ID_{50} (12 days) total	CF units per 0.1 ml.	CF units total
		ml.						
1.	Starting material No. 1	0.5	18	90	$10^{7.5}$	$10^{8.2}$	160	800
	Sucrose fraction							
	A	0.4	10	40	$10^{3.0}$	$10^{3.6}$	40	160
	B	0.6	18	108	$10^{3.0}$	$10^{3.8}$	40	240
	C	1.3	<5	<65	$10^{3.5}$	$10^{4.6}$	<10	<130
	D + pel.	1.4	<5	<70	$10^{5.0}$	$10^{6.1}$	<10	<140
2.	Starting material No. 2	1.0	30	300	$10^{5.5}$	$10^{6.5}$	40	400
	Sucrose fraction							
	A	0.9	10	90	$10^{2.5}$	$10^{3.5}$	20	180
	B	1.2	6	72	$10^{2.5}$	$10^{3.6}$	<10	<120
	C	2.6	<3.2	<83	$10^{2.5}$	$10^{3.9}$	<10	<260
	D + pel.	2.6	<3.2	<83	$>10^{4.5}$	$>10^{5.9}$	<10	<260
3.	Starting material No. 3	1.5	18	270	$10^{5.5}$	$10^{6.7}$	16	240
	Sucrose fraction							
	A	1.6	10	160	$10^{2.5}$	$10^{3.7}$	16	256
	B	2.4	10	240	$10^{3.0}$	$10^{4.4}$	8	192
	C	3.4	<1	<34	$10^{4.0}$	$10^{5.5}$	<4	<136
	D	4.0	<1	<40	$10^{5.5}$	$10^{7.1}$	<4	<160
	Pellet in saline	2.4	<1	<24	$10^{5.5}$	$10^{6.9}$	<4	<96
	45 per cent sucrose (no virus)		–		–		–	
	1:4 dilution in 11 per cent sucrose*		18		$10^{6.0}$		32	
	1:4 dilution in 37 per cent sucrose*		18		$10^{5.0}$		32	
	1:4 dilution in 56 per cent sucrose*		6.3		$10^{5.5}$		32	

* Titers expressed as dilutions of starting material.

cell-detaching factor as well as the soluble CF antigen localized in the upper fractions.

Since CF antigen activity tended to parallel cell-detaching activity in the previous sucrose density gradient centrifugation experiments, an additional experiment was conducted, using a narrower range of sucrose concentrations.

A 5-fold concentration (by volume) of type 2 adenovirus was made by half-saturation with ammonium sulfate, resuspension of the sediment in Hanks' solution, and dialysis overnight against Hanks' solution. Three sucrose density gradient tubes were prepared by layering 0.7 ml. each of 25, 20, 15, 10, and 5 per cent *w/w* sucrose per tube. The tubes were held overnight at 4°C., and then 0.5 ml. of the virus concentrate was placed on each sucrose column. The tubes were centrifuged as above, and the contents harvested in the same manner. The fractions were titrated for CF antigen and for cell detachment titer. The results are recorded in Table VI.

In fractions A, B, and C, the titers of cell-detaching activity and comple-

TABLE VI

Failure to Separate Cell Detaching Activity from Soluble CF Antigen by Sucrose Density Gradient Centrifugation

Material	Volume	Cell detaching titer	CF antigen titer	Ratio $\frac{\text{CF titer}}{\text{CDU}_{50}\text{ titer}}$
	ml.	*(CDU_{50}) per 0.1 ml.*		
Untreated virus		5.6	128	23
Concentrate—5 ×	1.5	18	512	28
Sucrose fraction A	2.6	5.6	128	23
B	1.8	5.6	128	23
C	1.8	1.8	32	18
D	2.4	<1	16	>16
E	2.8	<1	32	>32
Sucrose pellet in saline	1.5	<1	32	>32

ment-fixing antigen were in essentially the same ratio as in the starting material. The complement-fixing activity recovered in fraction E and the pellet possibly represents viral antigen, while that in fractions A, B, and C is the soluble antigen. The results of this experiment are interpreted as indicating that the cell-detaching factor and the soluble CF antigen are both smaller than the infectious virus particle and were not separated by the procedures used.

Test for Cell-Detaching Activity by Concentrated, Partially Purified Infectious Virus.—In an experiment to be reported in detail elsewhere (19), type 2 adenovirus infectious tissue culture fluid was concentrated and partially purified by acid-methanol precipitation and sucrose density gradient fractionation; the concentration factor was 40-fold by volume, and 10-fold by infectivity titration using six tubes per dilution. The concentrated preparation showed no detaching effect when tested undiluted, although in the same test the initial tissue culture fluid had a titer of 30 CDU_{50} per 0.1 ml.

Serum Neutralization of Cell Detachment

Cell-detaching activity of adenovirus suspensions was inhibited by human and rabbit antiserums, but only by relatively low dilutions of serum. The highest titers observed with human serums were 8, and type 2 rabbit antiserum with a neutralizing antibody titer of 320 neutralized type 2 cell detachment to a titer of 20. Pooled normal rabbit serum did not neutralize cell detachment at a dilution of 5 or 20, although it occasionally delayed cell detachment to some extent in comparison with cultures without added serum. Rabbit antiserums

TABLE VII

Homologous and Heterologous Neutralization of Cell-Detaching Activity by Rabbit Immune Serums

Rabbit antiserum (1:20)	Cell detachment in duplicate tubes			
	Adenovirus type 1	Adenovirus type 2	No virus	Uninfected control TC fluid
None	4; 4	3; 4	0; 0; 0; 0	0; 0; 0; 0
Normal rabbit serum	3; 4	3; 4	0	
Adenovirus type 1	0; 0	0; 0	0	
2	1; 0	0; 0	0	
3	4; 4	4; 4	0	
4	4; 4	3; 0	0	
5	4; 4	3; 2	0	
6	2; ±	2; 0	0	
Hemadsorption virus type 1	4; 4	4; 4	±	

which had been prepared by immunization with virus grown in HeLa or KB cells could not be tested at dilutions lower than 20, since the serum control tubes tended to detach, presumably owing to cytotoxic effects of antibody formed against the cells. Table VII presents results of a test in which type 1 and type 2 adenovirus infectious tissue culture fluids were tested against rabbit antiserums to adenovirus types 1 through 6 and an antiserum to an unrelated virus, a strain of hemadsorption virus type 1; the latter rabbit had been immunized with virus grown in HeLa cells, and the serum was included to control the specificity of the neutralization by adenovirus antiserums. Each serum was used at a dilution of 1:20, against 1.8 CDU_{50} of each virus. In conventional virus neutralization tests, each of the adenovirus antiserums was type-specific at a 1/20 dilution. As indicated in the table, cell detachment inhibition was not type-specific, although in this and other experiments, homologous rabbit antiserum consistently inhibited detachment. These results suggest that cell detachment neutralization is not suitable for rapid typing of adenoviruses.

The relationship of cell detachment inhibition to neutralizing and comple-

ment-fixing antibodies in human serums was investigated with serums of 51 children under 12 years of age; the majority of the children were under 4 years. All serums were tested for cell detachment inhibition against type 2 virus, and 34 were tested against type 1; the tests were made with a serum dilution of

TABLE VIII

Relation of Cell Detachment Inhibition to Virus Neutralizing and Complement-Fixing Antibodies in Serums of 51 Children

Type 1				Type 2			
Type 1 neutralizing antibody	CF antibody	Other adenovirus neutralizing antibody	Inhibition of cell detachment by type 1*	Type 2 neutralizing antibody	CF antibody	Other adenovirus neutralizing antibody	Inhibition of cell detachment by type 2
+	+	+ or −	10/10	+	+	+ or −	15/15
	−	+ or −	4/4		−	+ or −	4/4
−	+	+ or −	6/7	−	+	+ or −	13/14
	−	+	3/5		−	+	4/7
		−	0/8			−	0/11

* Numerator, No. of persons positive; denominator, No. of persons tested.

TABLE IX

Antibody Titers of Selected Children

Person	Adenovirus type against which tests were made	Antibody titers (reciprocal)		
		CF	Neutralizing	Cell detachment inhibiting
Br.	2	<2	<1	4
Ra.	1	<2	16	>2
Ma.	1	<2	16	4
D. Ty.	1	32	<1	2
K. Ty.	1	64	<1	2
Ca.	1	64	<4	<2
Cu.	2	≧32	64	8
Hi.	2	16	>64	8
We.	2	8	>64	4

1:2, or with 6 serums, 1:1. The serums were also tested for neutralizing antibody at a 1:4 dilution against types 1, 2, 3, and 5, and for complement-fixing antibody against types 1 or 2, at a serum dilution of 1:8. The results of Table VIII may be summarized as follows. With either serotype, those serums that were positive for neutralizing antibody also inhibited cell detachment by the homologous type; also, cell detachment was inhibited by a high proportion of serums having CF and/or heterologous neutralizing antibody but no homologous neutralizing antibody. In contrast, in children with no previous ade-

novirus infection, as indicated by the absence of CF antibody and neutralizing antibody to the 4 types tested, none of 19 tests was positive; this difference could not be attributed to difference in ages of children with and without adenovirus antibody. Twenty-two of the serums were also tested against type 5, with essentially identical results.

Additional evidence in support of the hypothesis that the antibodies inhibiting the cell detachment factor are distinct from neutralizing antibodies and antibodies reactive with the soluble antigen in CF was obtained from measurements of the corresponding activities of nine serums. In each instance, the three antibody titers were determined against the same adenovirus type. The results in Table IX show that in these serums there was no correlation between the titers of cell detachment inhibition and of neutralizing or complement-fixing antibody. From the results summarized in Tables VIII and IX, it is concluded that the inhibition of cell detachment is effected by a specific adenovirus antibody which is distinct from virus neutralizing and CF antibody.

DISCUSSION

The evidence presented here indicates that adenovirus infectious tissue culture fluid contains a trypsin-sensitive, non-dialyzable, adenovirus antigen capable of detaching HeLa cells, as well as certain other epithelial cells in continuous cultivation adhering to glass. Fractionation experiments have shown that the cell-detaching factor has a sedimentation coefficient smaller than that of the infectious virus.

The relationship to one another of the soluble complement-fixing antigen, the cell-detaching factor, and the material responsible for the early cytopathic effects is not fully defined. There is much evidence to suggest that the cell-detaching factor is not functionally identical with the soluble CF antigen. Although both are considerably smaller than the infectious virus particle, as indicated by the sucrose density gradient centrifugation experiments, they are not serologically identical, and the cell-detaching factor is more sensitive to trypsin than is the CF antigen. It has been possible to obtain preparations with high CF antigen activity, but without cell-detaching activity, either by trypsin treatment or by acid–45 per cent methanol precipitation, but the converse separation has not been obtained. It is conceivable that, in analogy with the LS antigen of vaccinia (20), CF and cell-detaching activity are functions of different moieties of the same molecule, with distinct antigenic sites.

The available evidence is insufficient to draw conclusions on the relationship or possible identity of the factors producing cell detachment and early cytopathic effects. It should be possible to determine their relationship by comparing the ratio of the two activities after a variety of treatments of crude tissue culture fluid. For this purpose it may be necessary to use different sets of HeLa cell cultures to demonstrate the two effects, since the system employed here was relatively reproducible for demonstrating cell detachment, but was generally

insensitive for demonstration of early cytopathic effects. On the other hand, cultures sensitive to the production of early cytopathic effects, *i.e.* very light cultures or cultures freshly changed to maintenance medium, were often unsuitable for cell detachment studies, in that control cultures often showed detachment. For study of the early cytopathic effects, the optimal system may be 24 to 48 hour observation of HeLa cell cultures, using inocula subjected to ultra-violet irradiation or gentle heating to prevent cytopathic effects associated with viral reproduction during the period of observation (18).

It appears somewhat unlikely that the cell-detaching factor represents fragments of degraded or incomplete adenovirus particles, since concentrated infectious virus was devoid of cell-detaching activity, and serologically the cell-detaching factor differed from the infectious virus.

The mode of action of the cell-detaching factor is not known, beyond the facts that the effect was temperature-dependent and the bulk of the factor was not irreversibly bound by the cells on which it acted.

The cell-detaching factor may play a role in the development of cytopathic effects in HeLa or KB cell cultures infected with adenoviruses, particularly with types 1, 2, 5. The close temporal relation between the time of appearance of cell-detaching factor and the onset of cytopathic effects in infected cultures (Table III) is in agreement with this hypothesis. Detachment of cells is a characteristic feature of the cytopathic effect, and the clumping of cells which is most characteristically seen with types 1, 2, and 5, could be due in part to surface changes produced by the cell-detaching factor.

Ginsberg (21, 22) has reported that HeLa cells infected with type 3 or 4 adenovirus do not undergo cytopathic changes when maintained in media containing high concentrations of human serum with or without homologous neutralizing antibody. In view of the lack of identity of the antibodies inhibiting cell detachment and virus infectivity, it appears possible that the former antibody may contribute to the suppression of cytopathic effects in this system.

As Boyer *et al.* (5) have pointed out in connection with the early cytopathic effects, the occurrence of non-viral cell injury complicates the interpretation of experiments in which large doses of virus are used in attempts to achieve synchronous infection. Also, comparison of the growth cycle of different adenovirus types following infection with large inocula must take into account the relative cell detaching activity of the different types, since it is quite conceivable that the changes induced by the cell-detaching factor could influence the growth cycle.

SUMMARY

Infectious tissue culture fluids of the majority of serotypes of adenovirus at low dilutions detach HeLa or KB cells from glass surfaces within a few hours after inoculation. A reproducible method for testing cell detachment was devised.

The factor present in infectious tissue culture fluids and responsible for cell detachment is trypsin-sensitive and non-dialyzable; it is smaller and more resistant to the effect of heat or ultraviolet light than the infectious virus particle.

Cell detachment activity was found to be temperature-dependent, and the cell-detaching titer of infectious tissue culture fluids was not affected by repeated exposure to HeLa cells. Inhibition of cell detachment by human or rabbit sera was observed only when other antibodies to adenovirus antigens were also present, but the antibody inhibiting cell detachment could not be correlated quantitatively with complement-fixing or homologous neutralizing antibody.

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