Studies of the Hemagglutinins of Type 3, 4 and 7 Adenovirus. (26523)

FRED ZUSCHEK* (Introduced by Oscar C. Liu)
Merck Sharp and Dohme Research Laboratories, West Point, Pa.

Rosen(1) showed that adenoviruses agglutinate erythrocytes of various species of animals selectively. The following work was initiated, in part, to confirm Rosen's observations and to study the properties of the hemagglutinin of Type 3 adenovirus (ADV-3). An attempt was made also to determine whether or not the hemagglutinin component of the adenovirus is a soluble substance.

Materials and methods. Virus. Viruses used in these studies were Types 3, 4, and 7 adenoviruses (ADV-3, 4, and 7) which were originally obtained from Dr. Heubner's laboratory. These viruses can be grown in either monkey kidney (MK) epithelial or HeLa cells. Most virus preparations were made in 1-liter or 5-liter Blake bottles containing confluent sheets of MK cells. Virus was harvested when cellular degeneration was complete, usually 5 to 7 days' incubation at 37°C. All virus pools used were prepared in absence of serum and stored at 4°C.

Concentration—Virus concentrates were prepared by methods described by Prier and LeBeau(2).

Antisera. Rabbit antisera against various types of adenovirus were prepared according to methods described by Rowe, *et al.*(3). Virus preparations used for immunizing rabbits were clarified by either low speed centrifugation or filtration through Seitz clarification pads to remove excessive cullular debris.

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Test—HA and HI tests were performed according to procedures outlined by Rosen(1), using as routine, erythrocytes obtained from the grivet or green monkey (*Cercopithecus aethiops*). Applicability of rhesus and guinea pig cells for these tests were also compared.

Complement fixation (CF) test. Concentrations of complement-fixing antigens were determined by the technic described by Osler, Straus, and Mayer(4), using the 50% hemolytic endpoint.

Centrifugation studies. All centrifugations were made with a No. 40 roter in the Model L Spinco at 4°C at speeds and times indicated.

Filtration studies. Filtrates were prepared by passing a minimum of 15 liters of harvested material through a Seitz-type, 6-inch diameter sterilizing pad. Sediment of harvested material was left undisturbed in the bottom of the glass container to prevent clogging of pads.

Separation of HA from CF activity by adsorption with Grivet Erythrocytes. Washed and packed erythrocytes were added to virus preparations to obtain a final concentration of 2%. Mixtures were agitated with a magnetic stirrer for 45 minutes at room temperature to keep the red blood cells in suspension. The mixture was then centrifuged at 2500 rpm for 5 min. Supernates were drawn off and subjected to the same procedure with fresh red blood cells. This cycle was repeated 3 times. Red blood cells were pooled, diluted 1:10 in distilled water. The cell suspension was frozen and thawed twice to lyse the cells. Lysates, as well as supernates, were tested for CF and HA activity.

Results. The data in Table I confirmed the results of Rosen(1) that Type 3 adenovirus (ADV-3) agglutinates erythrocytes obtained from either rhesus or grivet monkeys. Erythrocytes of the grivet were more sensitive to agglutination than rhesus erythrocytes. Adenovirus suspensions prepared from HeLa cells seemed to contain a greater concentration of hemagglutinin than those prepared from MK cells.

ADV 4 was also capable of agglutinating erythrocytes of green monkeys (Tables II and III). This finding was at variance with results obtained by Rosen(1). The difference may be due to individual variation of monkeys used as blood donors.

* Present address: 2538 S. E. 43rd St., Des Moines, Iowa.

TABLE I. HA Titration of ADV-3 Using Erythrocytes from Different Animals.

Virus preparation	Tissue culture source	HA titers* with erythrocytes from Guinea pig	Grivet monkey	Rhesus monkey
ADV-3	MK†	<4	16	8
" (100 × conc.)	MK	<4	512	64
"	HeLa	<4	2048	64
Control	MK	<4	<4	<4
"	HeLa	<4	<4	<4

* Titer expressed as reciprocal of highest dilution of virus in 0.5 ml of saline showing complete agglutination.

† MK—Rhesus monkey kidney cells.

Hemagglutinin of ADV-3 was fairly stable. (Table II). Activity was not completely destroyed by 1:4000 formalin and/or by storage at 4°C for as long as 2 years. As shown in the same table, hemagglutinins derived from ADV-4 and ADV-7 were also stable after storage at 4°C for over a year. Although original materials were not available for testing at the time of this study, HA titers of fresh MK-grown virus ranged from 64 to 128, and titers of fresh HeLa-grown virus, from 256 to 1024. Since these titers were about the same as HA titers of the adenoviruses mentioned in Table II, it is safe to assume that there had been no reduction of these titers after prolonged storage.

Attempts were made to follow inactivation of HA activity of ADV-3 at 56°C. HA titer dropped from 256 to 32 after 10 minutes at this temperature and remained constant (16 to 32) for the next 40 minutes. This indicated that either HA activity was moderately heat stable or that 2 types of hemagglutinins might be present in the preparation; one labile and one stable at 56°C.

Results of centrifugation studies shown in Table III indicated that HA activity was not sedimentable at speeds of 20,000 rpm for 60 minutes. However, at speeds of 36,000 to 39,000 for 90 to 180 minutes, HA activity was sedimented while most of the CF antigen usually remained in the supernatant fluids. These results indicated that the HA was a soluble component but different from the CF component. This difference was further emphasized in filtration studies. Data in Table IV showed that CF factor was partially removed by Seitz (S-1) sterilizing pads, whereas HA fractions appeared undiminished in filtrates.

Type specific rabbit antisera were prepared against adenoviruses, Types 1 through 7. HI tests with these sera against ADV-3, 4, and 7 showed that the homologous serum produced HI titers ranging from 1:512 to 1:1024, while the heterologous serum produced a titer of slightly over 1:8. Thus, in contrast to the CF reaction(3), the HI test appeared to be type specific, at least in the cases of types 3, 4, and 7.

Attempts were made to follow the production of HA and CF activity from infected HeLa and MK cell sheets at different time intervals. Table V summarized the results of this work. Inoculum used was a seed that produced infective titers rapidly, although the correlative infectivities were not shown. In MK cells HA activity appeared after 4 hours, but CF activity was not found until 23 hours, indicating another difference between these factors. CF and HA activities from the infected HeLa cells were found only 72 hours after infection, which was different from the HA production in MK cells. HA production by ADV-3 was also studied in 2 other human

TABLE II. HA Titers of ADV-3, ADV-4, and ADV-7 after Storage under Various Conditions.

Virus	Source	Storage conditions	HA titer*
ADV-3	MK	2 years at 4°C	128
"	"	Conc. with PVP and stored 1 year at 4°C	>256
"	"	Inactivated 6 days by 1:4,000 HCHO at 37°C and stored at 4°C >1 yr	8
"	"	Conc. with PVP (10×) and stored 1 year at 4°C	>256
ADV-4	"	Conc. with 20% Methanol on Celite and stored >1 year at 4°C	>256
ADV-7	HeLa	Stored at −20°C for 1 year	64

* Using Grivet monkey cells.

TABLE III. Effect of Ultracentrifugation on Hemagglutinins of ADV-3.

Virus	Speed and time	Original titer (HA)	(CF)	Titer of supernatant (HA)	(CF)	Titer of resuspended pellet (HA)	(CF)
HeLa	20,000 rpm/60 min.	2048		2048	—	128	—
"	20,000 rpm/90 min.	2048		1056	—	2048	—
MK	20,000 rpm/60 min.	8		16	—	4	—
"	40,000 rpm/90 min.	8		4	—	16	—
" with normal calf serum	39,000 rpm/90 min.	64		4	2	16	20
" without serum	39,000 rpm/90 min.	64		4	2	16	8 (AC)
MK 145	38,000 rpm/90 min.	16		0	—	32	—
MK	36,000 rpm/120 min.	512	3200	0	2000	320	40
"	36,000 rpm/180 min.	0	2000	0	1800	8	300

AC = anticomplimentary.

line cells (HB and Hep 2). HA titers of 1:32 to 1:64 were obtained from KB and Hep 2 cells 120 to 144 hours after infection with this virus.

Results summarized in Table VI showed that all of the HA activity was adsorbed by the grivet erythrocytes while most of the CF activity remained in the supernate and unadsorbed. An additional evidence of association was thus provided from soluble CF antigen common to all the adenoviruses. In certain experiments, low CF activity was found associated with red blood cell lysate. It was not determined whether this CF activity was identical with the soluble CF antigen.

Discussion. Our results confirm the original observation made by Rosen(1) regarding the capacity of adenoviruses, particularly ADV-3, to agglutinate erythrocytes obtained from grivet and rhesus monkeys. In our hands the grivet erythrocytes appeared to be more sensitive to agglutination by ADV-3 than those of the rhesus monkey. In contrast to Rosen's findings, these studies showed that ADV-4 and 7 also agglutinates the erythrocytes of these monkeys.

HA properties of ADV-3 were further studied. HA component of ADV-3 was separable from soluble CF antigen and from infectious virus. Centrifugation studies indicated that HA component may be larger than CF antigen but smaller than the infectious virus. HA activity of ADV-3 is separable from the infectious virus, as in vaccinia vi-

TABLE IV. Effect of Filtration on HA, CF, and $TCID_{50}$ Titers of Various Types of ADV.

Type	Pre-filtration HA	CF	$TCID_{50}$/ml*	Post-filtration HA	CF	$TCID_{50}$/ml*
3	8	7	5.9	8	<2	3.4
3	2	4	4.7	<2	<2	4.6
4	16	32	4.2	16	<2	3.6
4	32	8	4.5	32	2	3.9
4	16	16	4.1	>64	14	4.4
7	64	7	5.1	2	2	4.7
7	<2	<2	4.9	2	<2	4.6
7	<2	<2	4.4	2	<2	4.1

* Reciprocal of the dilution on Log_{10} showing 50% infective endpoint.

TABLE V. Production of HA and CF Antigens in Monkey Kidney and HeLa Cells Infected with ADV-3.

Hr after infection	Titers of virus from MK CF	HA	HeLa CF*	HA*
0	<2	<2	<2	<2
4	<2	2	<2	<2
6	<2	8	<2	<2
12	<2	16	<2	2
24	2	32	<2	<2
48	—	256	<2	<2
72	—	—	5	512

* Avg titers of 6 cultures.

TABLE VI. Separation of HA from CF Activity of ADV-3 by Adsorption with Grivet Erythrocytes.

ADV-3	Titers CF	HA
Original virus	1100	64
Supernate (post adsorption)	1120	<2
Erythrocyte lysate	216	—

ruses(7). On the other hand, filtration studies showed that HA component of adenovirus passed more readily through Seitz pads than CF antigen. Many factors might underline this discrepancy, *e.g.*, the difference in electrical charge on the particles or concentration of the different components.

HA activity was found earlier than CF antigen in fluids of MK cells infected with adenoviruses while the opposite usually obtains with viruses(8). This difference may reflect sensitivity of the tests or formation of each component at different times.

The increase in numbers of the adenovirus family has necessitated a search for a simple serologic test for identification. The value of CF test is rather limited, since it measures only a common soluble antigen, although modifications of this test by Pereira(5) and by Binn, *et al.*(6) have increased its usefulness. The specificity of the HI test with ADV-3, 4, and 7 Type 3 definitely indicates its value in diagnostic work. The HI test can be completed in less than 3 hours, thus it is relatively simpler than the CF test. Further, the reagents used in the HI are more stable than those used in the CF test.

Summary. Hemagglutinins of adenovirus Type 3, 4 and 7 were found to be separable from soluble CF antigen and infectious virus. Inhibition of HA activity with rabbit serum antibodies appeared to be type specific. The HA component was relatively stable to heat, formalin and storage at 4°C. It was more readily filterable than CF antigen and was produced earlier than the CF in infected MK cells.

1. Rosen, L., *Virology,* 1958, v5, 574.
2. Prier, J. E., LeBeau, R. W., *J. Lab. & Clin. Med.,* 1958, v51, 495.
3. Rowe, W. P., Huebner, R. J., Bilmore, L. K., Parrot, R. H., Ward, T. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 570.
4. Osler, A. G., Strauss, J. H., Mayer, M. M., *Am. J. Syphilis, Gonorrhea & Venereal Dis.,* 1952, v36, 140.
5. Pereira, H. G., *J. Path. & Bact.,* 1956, v72, 105.
6. Binn, L. N., Hilleman, M. R., Rodriguez, J. E., Glabere, R. R., *J. Immunol.,* 1958, v80, 501.
7. Burnet, F. M., Boake, W. C., *ibid.,* 1946, v53, 1.
8. Henle, W., *Advances in Virus Research,* 1953, v1, 141.

Received January 11, 1961. P.S.E.B.M., 1961, v107.

Lipid Metabolism in Cultured Cells. I. Factors Affecting Cholesterol Uptake.* (26524)

J. MARTYN BAILEY (Introduced by C. R. Treadwell)
Department of Biochemistry, School of Medicine, George Washington University, Washington, D. C.

The contribution of serum lipids to the nutrition of mammalian cells in culture has been described by Bailey *et al.*(1). It was shown that cells utilize triglycerides most rapidly, but also take up considerable quantities of cholesterol.

Rutstein *et al.*(2) reported that explants of human aorta accumulate cholesterol from a serum growth medium. It was claimed that cholesterol uptake was inhibited by addition of polyunsaturated fatty acids. A number of cell strains synthesize cholesterol *de novo* from simpler constituents in the medium(3). With an adequate supply of serum lipids, however, most of the cell cholesterol is derived from the serum(1). In view of the implication of cholesterol desposition in the etiology of atherosclerosis, it was of interest to determine the factors affecting cellular cholesterol uptake in an *in vitro* system.

Materials and methods. Preparation of growth media and propagation of the MB III strain of cells of mouse lymphoblasts has been described(1). Growth medium used for propagation of stock cultures was human

* This work was supported in part by a research grant from Nat. Heart Inst., Nat. Inst. Health, U.S.P.H.S.