Hemagglutination and Hemagglutination-Inhibition with Adenovirus Type 12.* (29799)

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The adenoviruses of human origin have been classified into groups on the basis of their hemagglutination reactions(1,2). Viruses in Group 1 (types 3, 7, 11, 14, 16, 20, 21, 25 and 28) agglutinate only rhesus or grivet monkey erythrocytes. Members of Group 2 (adenovirus types 8, 9, 10, 13, 15, 17, 19, 22, 23, 24, 26 and 27) agglutinate rat erythrocytes; however, some of these types also agglutinate monkey erythrocytes, although to a lower titer. The adenoviruses classified in Group 3 (types 1, 2, 4, 5 and 6) partially agglutinate rat cells, but agglutination patterns are complete or almost complete in the presence of heterotypic immune serum. Adenovirus types 12 and 18 have been classified into a fourth group on the basis of the fact that hemagglutinins have not been demonstrated for these two immunotypes.

We have recently observed that adenovirus type 12 may agglutinate rat erythrocytes, and that hemagglutination may be specifically inhibited by homotypic immune serum.

Materials and methods. Virus strains. The prototype Huie strain of adenovirus type 12 was received in 1958 from Dr. Robert J. Huebner at its 15th passage level (13 in HeLa or KB cells and 2 in HeLa cells). This strain was passaged an additional 3 to 5 times in HeLa cells for use in these studies. In addition, an adenovirus type 12 preparation designated as strain #5184 was obtained from Dr. Huebner at its 4th passage level (2 in KB cells and 2 in human embryonic kidney cells). Two non-prototype adenovirus type 12 strains were received from Dr. Leon Rosen; these, designated as strains 17357 and 97838, were passaged twice in KB cells and once in HeLa cells in this laboratory.

Hemagglutinating antigens. Adenovirus hemagglutinating antigens were prepared in 200 ml bottle cultures of HeLa cells of the D line. Cultures were infected with 0.5 ml of a 1×10^{-1} dilution of virus. During viral multiplication, the cultures were maintained on 8 ml of a medium consisting of 5% chicken serum, 45% Medium 199 and 50% lactalbumin hydrolysate-yeast extract medium.† The infected cultures were incubated at 36°C and harvested when the cells showed 4-plus degeneration. The materials were stored at —20°C, and prior to use as antigens were clarified by centrifugation at 1500 rpm for 5 minutes.

Adenovirus immune sera. Immune sera to the adenoviruses were prepared in rabbits as described elsewhere(3). In addition, reference immune sera (rabbit) to adenovirus types 12 and 18 were obtained from Dr. Morris Schaeffer and Dr. Robert J. Huebner.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests. Tests were performed by the microtiter method(4) in disposable "V" plates.‡ Erythrocytes from rhesus monkeys or rats (Sprague-Dawley strain) were collected in Alsever's solution, and were washed 3 times with physiological saline prior to standardization of the cell suspension. A 1% suspension of each erythrocyte type was prepared in physiological saline; this suspension was standardized with a Coleman Jr. spectrophotometer and the concentration was adjusted to give an O.D. at 490 mμ of 0.40 for monkey erythrocytes or 0.30 for rat erythrocytes. For tests with adenoviruses of Group 2, normal, inactivated rabbit serum was added to the rat cell suspension to give a dilution of 1:50 or 1:100; this prevents spontaneous agglutination of the rat erythrocytes(1). For tests with adenoviruses of Group 3, immune rabbit serum to

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† The lactalbumin hydrolysate-yeast extract medium has the following composition: lactalbumin hydrolysate, 0.50%; glucose, 0.45%; yeast extract, 0.10%; sodium bicarbonate, 0.11%. These components are dissolved in Earle's balanced salt solution.

‡ Cooke Engineering Co., Alexandria, Va.

adenovirus type 6 (or another immunotype of Group 3 if type 6 was the test virus) was added to the rat cell suspension to give a concentration of 1:50 or 1:100; hemagglutination with adenoviruses in Group 3 is indirect in nature and occurs in the presence of immune serum(1,5).

Physiological saline was employed as a diluent for both virus and sera.

Adenoviruses to be assayed for HA activity were dispensed in 0.025 ml volumes into three sets of 2-fold dilutions, and 0.025 ml of physiological saline was added to each dilution. To one set of virus dilutions was added 0.025 ml of a suspension of rhesus monkey erythrocytes, to one set was added 0.025 ml of the rat erythrocyte suspension containing normal rabbit serum, and to the third set of dilutions was added 0.025 ml of the rat cell suspension containing immune serum to a Group 3 adenovirus type. Appropriate controls on each cell suspension were included. Tests were incubated at 37°C for 45-60 min, and the highest virus dilution producing complete agglutination of the erythrocyte suspension was considered the HA titer. (In some instances adenoviruses of Group 3 produce an agglutination pattern in which there is a small button of cells in the tip of the "V" cup, surrounded by a large ring of agglutinated cells. However, agglutination of this type is distinctive and endpoints are easily determined.) For use in HI tests viral preparations were diluted to contain four HA units in a volume of 0.025 ml. Sera to be examined in HI tests were treated with kaolin(1) or filtrates from a psychrophylic *Pseudomonas* sp. (Ps. filtrates)(6) for removal of nonspecific inhibitors, and they were also absorbed with the test erythrocytes to remove natural agglutinins.

Two-fold dilutions of serum to be assayed for HI antibodies were dispensed in 0.025 ml volumes, and to each dilution was added 0.025 ml of virus diluted to contain 4 HA units. A serum control consisting of serum dilutions and 0.025 ml of saline was included to test for possible agglutinating activity of the serum alone. Also, a check on the antigen unitage of each test virus was included. The serum-virus mixtures were incubated at room temperature for one hour, after which 0.025 ml of the appropriate cell suspension was added and the tests were incubated at 37°C for 45-60 minutes. The HI titer of the serum was the highest dilution completely inhibiting hemagglutination by the 4 units of antigen.

Neutralization tests. Neutralizing antibody assays for adenovirus type 12 were conducted in human fetal diploid kidney cell cultures. Sera were inactivated at 56°C for 30 min, and 2-fold dilutions were examined for their ability to inhibit the cytopathic effect of 100 TCD_{50} of virus. Tests were read after 7 days' incubation at 36°C.

Results. Hemagglutination with adenovirus type 12. Table I shows some representative HA titers of various adenovirus type 12 preparations with 3 different erythrocyte suspensions (rhesus monkey, rat with normal rabbit serum and rat with adenovirus type 6 immune serum). The virus lots examined included a stock lot of adenovirus type 12 antigen (Huie strain), some clones of adeno-

TABLE I. Hemagglutination with Adenovirus Type 12.

Virus strain	Lot No.	Hemagglutinating titers with: Rhesus cells	Rat cells with normal rabbit serum	Rat cells with adeno 6 immune serum
Huie (stock lot)	TC 14132	<1:2	<1:2	1:8
" (clone)	Plaque 518	<1:2	<1:2	1:16
" "	" 526	<1:2	<1:2	1:16
" " *	" 487	<1:2	<1:2	1:8
" " *	" 489	<1:2	<1:2	1:8
5184†	—	<1:2	<1:2	1:8
17357‡	TC 9886	<1:2	<1:2	<1:2
97838‡	TC 9885	<1:2	<1:2	1:4

* Isolated from ultraviolet-irradiated preparation.
† Obtained from Dr. Robert J. Huebner.
‡ Originally obtained from Dr. Leon Rosen.

virus type 12 isolated from the prototype Huie strain, the #5184 virus strain from Dr. Huebner and 2 nonprototype strains of adenovirus type 12 (strains 17357 and 97838) obtained from Dr. Leon Rosen. The clones were isolated by Dr. N. Takemori of this laboratory by plating irradiated or unirradiated adenovirus type 12 preparations (strain Huie) on monolayers of human embryonic kidney cells (3rd passage); plaques were picked and subpassaged in KB cells. The nonprototype adenovirus type 12 strains from Dr. Rosen were also subpassaged twice in KB cells and once in HeLa cells in this laboratory.

All of the adenovirus type 12 preparations derived from the prototype strain were found to agglutinate rat cells in the presence of immune serum to adenovirus type 6 (or immune serum to other adenovirus types of Group 3). HA titers were not high, but they were reproducible. The agglutination pattern seen with adenovirus type 12 preparations was that often seen with viral types of Group 3, *i.e.*, a small button of cells surrounded by a large ring of agglutinated cells. One of the 2 nonprototype adenovirus type 12 strains from Dr. Rosen could not be shown to hemagglutinate any of the erythrocyte suspensions, but the other had a titer of 1:4 with rat cells in the presence of heterotypic immune serum. The #5184 adenovirus type 12 strain received from Dr. Huebner had a titer of 1:8 with rat cells in the presence of heterotypic immune serum.

The need for selecting donor animals with erythrocytes agglutinable by adenoviruses has been emphasized by Rosen(1), but he found that if an individual animal's cells were agglutinable by one member of an adenovirus group they were suitable for use with other immunotypes of the group. However, in these studies it was found that rat erythrocytes agglutinable by viruses of Group 2 or 3 were not necessarily agglutinated by adenovirus type 12. Thus, of 8 donor rats whose cells were agglutinable by adenoviruses of Group 2 and 3, the cells from one animal were not agglutinated by adenovirus type 12, and those from another showed only a trace of agglutination. Erythrocytes of the other 6 rats were satisfactorily agglutinated by adenovirus type 12, and no differences in HA titer of the virus were observed with cells from the 6 different rats.

TABLE II. Hemagglutination - Inhibition with Adenovirus Type 12, Huie Strain.

Adenovirus serum type	Lot No.	Hemagglutination-inhibition titer *vs* adenovirus type 12	
		Kaolin-treated serum	Ps. filtrate-treated serum
1-11	—	<1:10	<1:10
12	1306-1	1:40	1:80
"	1306-2	1:40	1:160
"	MBA 3-293*	1:80	1:160
"	T12, lot 1†	1:80	1:160
13-17	—	<1:10	<1:10
18	1291	<1:10	1:10
"	1292	<1:10	<1:10
"	RAS 3-136*	<1:10	1:10
"	T18, lot 1†	<1:10	<1:10
20, 21, 24-26, 30	—	<1:10	<1:10

Reference immune serum obtained from:
* Dr. Robert J. Huebner.
† Dr. Morris Schaeffer.

Hemagglutination-inhibition with adenovirus type 12. Various adenovirus immune sera were examined for their ability to inhibit HA by the Huie strain of adenovirus type 12. Two different methods were employed for removal of nonspecific inhibitors of hemagglutination, *viz.*, kaolin adsorption (1) and treatment with *Pseudomonas* filtrates(6). Sera treated by these two procedures were examined in parallel against 4 HA units of adenovirus type 12 antigen.

In Table II, it is seen that immune sera to adenovirus types 1-11, 13-17, 20, 21, 24-26 and 30 failed to inhibit HA by the Huie strain at serum dilutions of 1:10 or greater. However, HA was inhibited by all of the adenovirus type 12 immune sera examined. These included sera prepared in this laboratory from the prototype Huie strain and reference immune sera from Dr. Morris Schaeffer and Dr. Robert J. Huebner. HA by the 97838 adenovirus type 12 strain and the adenovirus type 12 #5184 strain from Dr. Huebner was also specifically inhibited by type 12 immune sera. Two of the type 18 sera treated with Ps. filtrate inhibited HA by adenovirus type 12 (Huie strain) to a

titer of 1:10. These low titers are of interest in view of the fact that adenovirus type 12 and 18 show a minor antigenic relationship in neutralization tests.

The HI titers of the Ps. filtrate-treated sera tended to be higher than those of kaolin-treated sera; other studies in this laboratory(6) have shown that the former treatment removes less specific antibody for the echoviruses and reoviruses than does kaolin adsorption.

The identity of our stock lot of hemagglutinating type 12 adenovirus was further confirmed by examining it in neutralization tests with adenovirus type 12 immune serum prepared in this laboratory and with type 12 reference immune serum from Dr. Huebner. The serum prepared in this laboratory had a neutralization titer of 1:320 against 100 TCD_{50} of the virus, and the reference immune serum (MBA-3-293) had a neutralizing antibody titer of 1:640 against the virus.

Discussion. Adenovirus type 12 would not appear to be distinctive by virtue of its lack of a hemagglutinin; results of these studies suggest that it might be classifiable into Group 3, as it agglutinates rat erythrocytes in the presence of heterotypic immune sera (to members of adenovirus Group 3).

The HA titers of adenovirus type 12 preparations were low, and this might explain difficulties in showing HA activity with this viral type. (Infectivity titers were also low, ranging in the vicinity of 10^{-2} TCD_{50} per 0.1 ml when the virus was titrated in human fetal diploid kidney cell strains.) Another possible explanation for difficulties in demonstrating HA might lie in the fact that erythrocytes agglutinable by other adenovirus types are not necessarily agglutinated by adenovirus type 12 (although most of our donor rats with cells suitable for use with adenoviruses of Group 3 were also suitable for use with adenovirus type 12). It is also possible that the passage history of the prototype virus in different laboratories might have an influence on its HA capacity. The 97838 strain from Dr. Rosen failed to hemagglutinate when examined at its second passage level in KB cells, but after subpassage in HeLa cells hemagglutinins were demonstrable.

Whether HA is a characteristic of only certain strains of adenovirus type 12 remains to be determined. The 17357 strain did not show HA after passage in either KB or HeLa cells, but it is possible that the examination of more concentrated preparations might reveal the presence of hemagglutinins.

The demonstration of HA and HI with adenovirus type 12 is also of interest in that it offers another immunological tool for use in the examination of tumor-bearing animals for the presence of adenovirus type 12 antigen or antibody.

Summary. Hemagglutination has been demonstrated with the prototype Huie strain and two nonprototype strains of adenovirus type 12. This viral type resembles those classified in Group 3 in that hemagglutination occurred with rat cells in the presence of heterotypic immune serum. Hemagglutination was specifically inhibited by immune serum to adenovirus type 12, but not immune serum to other adenoviruses. The identity of the hemagglutinating virus as adenovirus type 12 was confirmed by both hemagglutination-inhibition and neutralization tests with reference immune sera prepared in other laboratories.

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