Hemagglutination with Adenovirus Serotypes Belonging to Rosen's Subgroups II and III[1] (34227)

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Adenovirus serotypes belonging to subgroups II and III give a complete and partial agglutination of rat erythrocytes, respectively (1). The latter kind of hemagglutination (HA) can, however, at times be difficult to detect. Naéz *et al.* (2) reported that even some strains of adenovirus serotypes belonging to subgroups II and III were devoid of hemagglutinating capacity. Similar observations led Bauer and Wigand (3) to question the applicability of the hemagglutination–inhibition (HI) test for typing of adenovirus isolates.

In recent studies, the different complete HA's of adenovirus serotypes belonging to Rosen's subgroup III have been identified as virions (4, 5), empty capsids (4), dodecons (representing the only soluble complete HA of serotype 4) (4, 6, 7), and aggregates of two pentons or of two fibers (serotypes 1, 2, 5, and 6) (5, 8, 9). All these polymers of structural components represent complexes, which are at least divalent with respect to their capacity to interact with receptors on red cells. On the other hand monovalent components, *i.e.*, isolated pentons and fibers, were found to represent incomplete HA's (5, 6, 8). The fact that preparations of members of Rosen's subgroup III contain relatively more incomplete than complete HA's (5, 6, 8) was proposed as a possible explanation for the partial agglutination given by these serotypes. In the following results from further studies of this problem will be presented. The nature of the interaction between fibers of some members of subgroups II and III (types 1, 2, 4, and 15) and human O erythrocytes described by Kasel *et al.* (10, 11) was also studied. Finally, an examination was made of the relative usefulness of different forms of HA's as antigen in hemagglutination–inhibition (HI) tests.

Methods. Virus materials. Prototype strains of adenovirus types 1, 2, 3, 4, 5, 6, 9, 10, 11, and 15 were propagated in monolayer cultures of MAS-A cells, a human bone marrow cell line, or a human embryonic lung cell line, Lu 106, and concentrated by use of the techniques previously described (5). The type specificity of the strains used was controlled in HI tests by use of reference sera obtained from the Department of Health, City of New York, N. Y. and kindly made available by Dr. Åke Espmark. A pronounced cross reactivity was observed between serotypes 9 and 10 as previously reported (12).

Separation of virus specific components. Virions to be used as HA antigen, were prepared by centrifugation in discontinuous CsCl gradients (7). However, preparations used for immunizations were purified further by banding twice in density gradients of CsCl. Soluble components were isolated by fractionation of the supernatant obtained after three consecutive centrifugations at H Performance index (Pi) 5.3 (20,000 rpm rotor 40, Spinco division Beckman Instruments Inc., California). The structural components used for production of antisera and in some cases for different experiments were prepared in the following way. The dodecons of serotype 4, the dimers of pentons of serotype 6, and the monomeric pentons of serotypes 2 and 4 were isolated by exclusion chromatography

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on agarose Bio-Gel A15m (Bio-Rad Laboratories, Richmond, California). The aggregates of fibers of serotype 2 (8, 9) were obtained by anion exchange chromatography on DEAE Sephadex A25 (Pharmacia Fine Chemicals, Uppsala, Sweden) and a subsequent separation by exclusion chromatography on Bio-Gel A15m. The fibers of serotype 2 were purified by chromatography on DEAE Sephadex A25 (8) followed by isoelectric focusing to its isoelectric point 6.1 (13, 14, Wadell, unpublished) and finally exclusion chromatography on Sephadex G100 (4). Fibers of serotype 4 were separated on Bio-Gel A15m and subsequently purified by anion exchange chromatography on DEAE Sephadex A25 (4) whereas fibers of serotype 6 were purified by use of the latter technique only. However, all monomeric pentons and fibers of serotypes 1, 2, 4, 5, 6, and 15 used as incomplete HA's in serological and interference tests were prepared by rate zonal centrifugation in sucrose gradients at Pi 6.5–7.0 for 26 hr (SW25, Spinco) (7).

Determination of biological activities. Hemagglutination and hemagglutination–inhibition tests were performed both in tubes (12 × 75 mm) as was previously described (5, 15) and in disposable microtiter U-plates (Linbro Chem. Co. Inc., New Haven, Connecticut). Due to their fragility, pools of erythrocytes from three to five rats were used only on the day of collection. It was however noted that samples of blood which had been stored in physiological saline for some days at 4° and therefore were severly hemolyzed, retained their capacity to become agglutinated by adenoviruses without complicating spontaneous agglutination. Human O erythrocytes stored at 4° in Alsevers' solution were stable for more than 1 week. Incomplete HA's, *i.e.*, fibers and pentons, were demonstrated in hemagglutination–enhancement (HE) tests by incubation with heterotypic antisera. The sera used for indication of incomplete HA were chessboard titrated and diluted to contain 2 HE units (HEU). One HE unit was equivalent to the last dilution step of antiserum or incomplete HA, in which a distinct agglutination of red cells was prevalent. Fibers of serotypes belonging to subgroup III contain a subgroup-specific antigen (6, 16) and were demonstrated by an antiadenovirus type 5 serum, except in the case of serotype 5 fibers which were incubated with an antiserotype 6 serum. Part of the vertex capsomer is group-specific (5, 15). On the basis of this fact pentons were indicated by an antiserotype 3 dodecon serum. The Takatsy microtechnique for complement fixation (CF) was performed as previously described (5).

Test for interference by fiber incomplete HA with the activity of virion-associate complete HA. Both a macro- and a microtechnique were applied in these experiments. The data for the latter are given in parentheses. In order to simulate the condition in unfractionated virus materials fiber incomplete HA of serotypes belonging to subgroups II and III containing 320–640 HEU/0.2 ml was titrated in serial twofold dilutions of 0.2 (0.025)-ml volumes of PBS (0.067 *M* phosphate buffered physiological saline, pH 7.2). Another 0.2 (0.025) ml containing 2 HAU of virions were added to all tubes (cups). Subsequently 0.2 (0.05) ml of 0.5 (0.75)% rat or human O erythrocytes were added. The tests were read after sedimentation of the erythrocytes at 37°. The last dilution in which no HA activity could be observed was taken as end point.

Adsorption of purified virions on rat and human red cells. Samples (0.2 ml) of various dilutions of virions in physiological saline were adsorbed in glass tubes (4 × 50 mm) with packed erythrocytes representing different concentrations. The tubes were shaken mildly four times during an adsorption for 1 hr at 37°. The erythrocytes were then removed by centrifugation at 1500 rpm for 10 min and the supernatants were tested for residual HA activity by the microtechnique.

Hyperimmune sera. Preparation. Rabbits were injected intramuscularly with 4 ml of virus material mixed with Freunds complete adjuvant. Four to 5 weeks later an intravenous booster of 1 ml was given. The animals were bled after another week. *Antigens.* Unfractionated preparations of adenovirus types 2, 4, and 6 were concentrated 10–20 times by forced dialysis against poly-

TABLE I. HA and HE of Rat Cells with Unfractionated Virus Preparations.

Adenovirus type	HA (units/0.4 ml)	Penton-specific HE (units/0.4 ml)[a]	Penton plus fiber-specific HE (units/0.4 ml)[b]	Hexon-specific CF (units/0.025 ml)[c]
2	7[d]	12	13	8
4	8	9	11	8
6	10	13	15	10
9	12	12	12	8

[a] Demonstrated by incubation with an antiadenovirus type 3 serum.

[b] Demonstrated in the presence of an antiadenovirus type 5 serum.

[c] Determined by an antiadenovirus type 9 virion serum, and in the case of type 9 by an antiadenovirus type 3 virion serum.

[d] All titers are expressed in $\log_2$ values.

ethylene glycol 20 *M* (Kebo, Stockholm, Sweden). Structural components of these serotypes were purified as described above. *Specificity*. All antisera displayed only homotypic reactions in HI tests. The antifibersera reacted neither with hexons and extracts of uninfected host cells in CF tests nor with pentons of serotype 11 in HE tests. The antipenton sera, however, displayed group-specific hexon antibodies at comparatively low dilutions.

Results. Hemagglutination enhancement (HE) of unfractionated virus preparations. An excess of incomplete HA's compared to complete HA appeared to be prevalent among adenovirus types belonging to subgroup III (4, 5, 8), whereas the opposite relationship was found in preparations of subgroup II members (17, 18).

The relative variation in the enhancement of HA activity by heterotypic antisera observed for some representative serotypes are shown in Table I. The serotypes chosen in this presentation all have virion-associated HA but differ in the kind and relative quantity of soluble complete HA's. In preparations of serotype 2 the aggregates of fibers dominate over the dimers of pentons (8), whereas adenovirus type 6 displays the reverse relationship between these two kinds of soluble complete HA's. Dodecons represent the only demonstrable soluble complete HA in materials of serotype 4, whereas the representative of subgroup II, serotype 9, contains both dodecons and aggregates of fibers. A strong enhancement of the HA activity was obtained with all tested members of subgroup III (cf. Fig. 1) and the sedimentation patterns of the rat red cells changed from a partial towards a more complete agglutination. A prozone of negative bottom patterns remained however (Fig. 1 test B) possibly indicating that the relative excess of incomplete HA's could interfere with the agglutinability of red cells by complete HA's (Fig. 1 test A). It might be mentioned that the agglutination patterns were most distinct after incubation at temperatures below 20°. The HA titer of subgroup II members was not significantly changed by the presence of heterotypic antisera.

Serotype 9 causes complete agglutination of human O red cells (19). No such activity has previously been observed with serotypes belonging to subgroup III. However, fibers of some of these serotypes have been demonstrated to interact with human red cells (10, 11). Against this background, it was decided to test whether these serotypes and also other members of subgroup II and III, previously not described to interact with human red cells, possibly could agglutinate these cells in the presence of heterotypic antisera. These tests revealed that all serotypes studied (types 1, 2, 4, 5, and 6, members of subgroup III, and 9, 10 belonging to subgroup II) gave a clearly distinguishable agglutination under these conditions (see Fig. 1, test E). Titers obtained with various serotypes in tests with both human O and rat red cells were of a corresponding order of magnitude (cf. Fig. 1). Type 15 represents an exception. Crude virus preparations of this serotype displayed no HA activity with human O

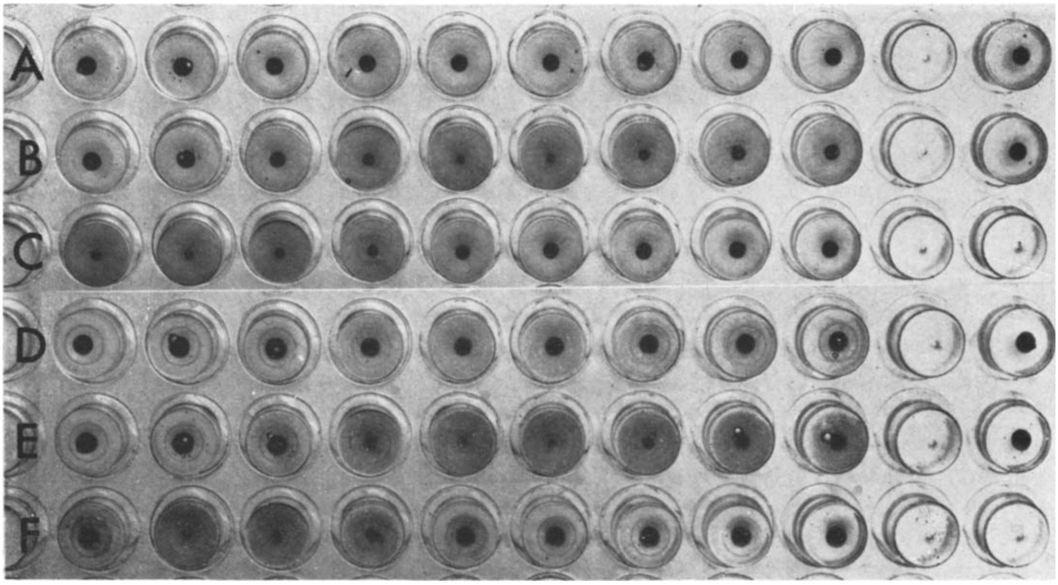

FIG. 1. Agglutination of rat (A–C) and human O (D–F) erythrocytes by an unfractionated adenovirus type 2 preparation: (A, D), HA titration in twofold dilution steps from 1/10 to 1/2560 plus a negative control of the erythrocytes. (B, E), Hemagglutination–enhancement (HE) in the presence of an antiadenovirus type 6 serum at the same dilutions as under A, D. (C, F). Titration at the same conditions as under B, E from dilutions of 1/80 to 1/20, 480.

cells even at low dilutions but hemagglutination was demonstrated with heterologous antisera to an end point 8–16 times lower than that noted with rat red cells.

Hemagglutinating activity of purified virions. The capacity of virions of serotypes 1, 2, 4, 5, 6, 9, and 15 to agglutinate rat and human O erythrocytes were compared. As expected serotypes 9 and 15 caused complete agglutination of rat erythrocytes. The bottom patterns observed with types 1, 2, 5, and 6 were also complete or almost complete (Fig. 2B), whereas virions of serotype 4 gave partial agglutination. In addition to this agglutination previously demonstrated by Bauer and Wigand and in this laboratory (4, 5, 20) similar results were also obtained with human O red cells although in this system more pronounced prozone phenomena were recorded. No difference in the end points obtained with erythrocytes of rat or human O origin was observed for any of the types, except serotype 15. In this case the titer recorded with rat erythrocytes exceed the end point with human red cells by 6–7 twofold dilution steps. The agglutination patterns of

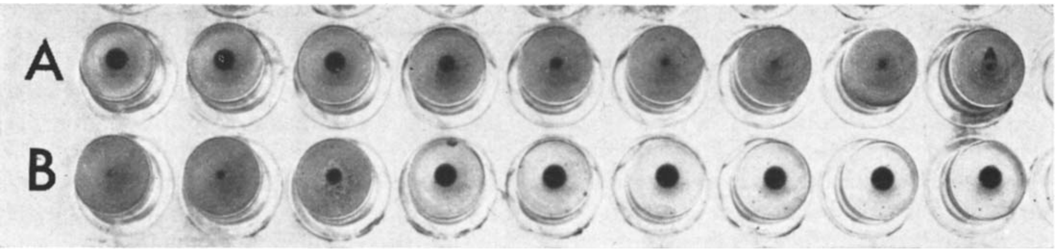

FIG. 2. Agglutination of rat red cells by virions of adenovirus type 6 in the presence of different amounts of heterologous fibers: (A), Adenovirus type 2 fibers with an initial concentration of 2560 HEU/0.4 ml were titrated in serial twofold dilutions. Two HAU of virions were added to all cups. (B), Virions titrated in PBS in twofold dilution steps.

TABLE II. The Capacity of Homologous and Heterologous Adenovirus Fibers to Interfere with the Hemagglutinating Activity of Virions.

		Types of virions						
		Subgroup II		Subgroup III				
Fibers of types	Type of erythrocytes	9	15	1	2	4	5	6
1	Rat	<1	<1	2	1	4	2	1
	Human O	8[a]	4	8	16	32	32	32
6	Rat	<1	<1	2	1	2	4	4
	Human O	2	8	2	2	8	8	16
15	Rat	<1	<1	1	1	2	1	1
	Human O	2	8	4	4	8	4	8

[a] The highest dilution of fibers, which completely prevented the activity of 2 HAU of virions.

the latter cells were only partial even when freshly prepared virions of serotype 15 were used.

Agglutinability of human O red cells obtained from different donors. Erythrocytes from 20 human O donors were used in HE tests indicating all incomplete HA's present in unfractionated preparations of serotypes 2, 4, and 5. The titers obtained with cells of different donors did not vary more than one twofold step of dilution. However, bottom patterns of agglutinated cells varied markedly. With the same preparation of serotype 2 a range of bottom pictures from partial to the almost complete agglutination demonstrated in Fig. 1 was noted. Serotype 15 produced a range of patterns from hardly discernable agglutination, indicated only by the failure of the erythrocytes to slide down when the rack was tilted, to clear-cut partial agglutination. The agglutination patterns of human O cells by serotype 4 were intermediate to those observed with the other two serotypes. It should be noted that the degree of agglutinability of cells from different donors varied to the same extent for the various adenovirus types tested. Because of the variation in agglutinability of cells, pools of erythrocytes from at least five human or rat donors were used in the adsorption and interference experiments to be described below.

Studies on competitive interaction between incomplete HA's and virion-associated complete HA and red cells. The relative capacity of incomplete HA's to interfere with the interaction of a complete HA and red cells was studied with different members of subgroups II and III. The macro- and microtechniques used were presented under "Methods". As a model system the competitive influence of fiber incomplete HA on the agglutinating activity of virion-associated complete HA was analyzed. Fibers of serotypes 1, 2, 4, 5, and 6 (subgroup III) and 15 (subgroup II) were tested against preparations of virions of these serotypes and in addition of serotype 9.

Shortage of the reagents in certain cases, necessitated the use of a microtechnique (see "Methods") in the experiments comparing the extent of interference obtained with rat and human O red cells. It should be mentioned that the macrotechnique resulted in 4- to 8-fold higher interference titers than the microtechnique which was used in the experiment described in Table II. In order to increase the simplicity of presentation, Table II only contains the results of interference mediated by fibers of three representatives of the different serotypes tested. The modification of the agglutinability of human O red cells by fibers of all serotypes studied was more marked than the corresponding effect on rat erythrocytes. The order of magnitude of this difference in tests with subgroup III virions and red cells was relatively higher for fibers of serotypes 1, 2, and 5 as compared to those of types 4, 15, and 6. It should be noted that the hemagglutination of rat red

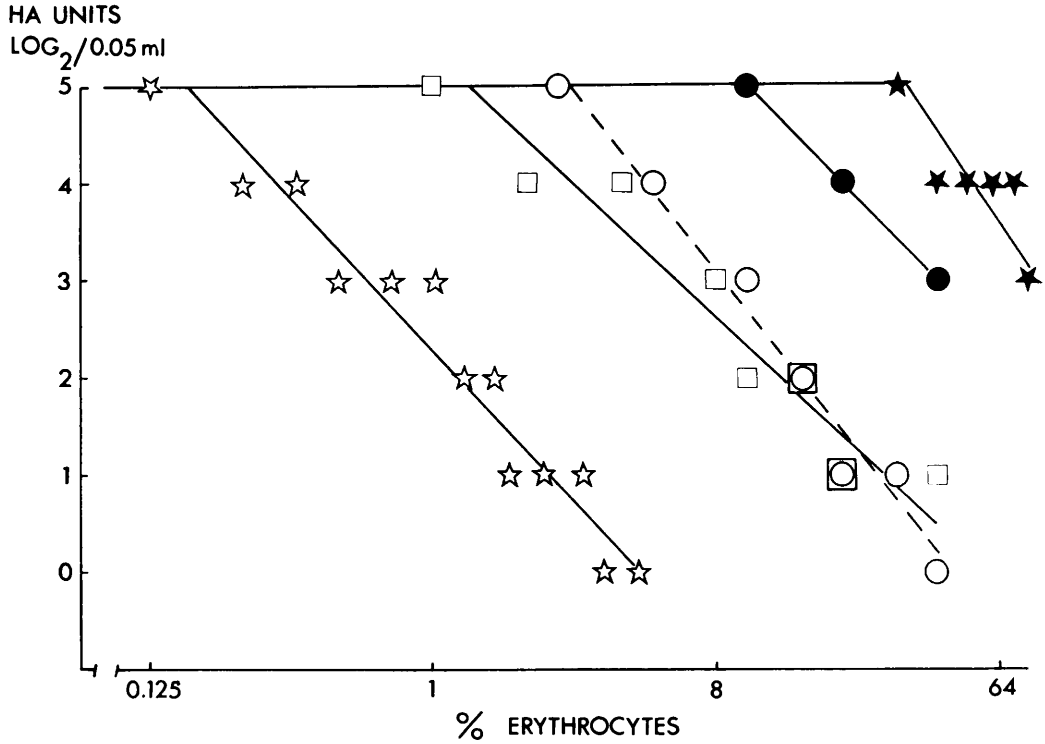

FIG. 3. Hemagglutinating activity remaining after adsorption of a constant amount of purified virions (type 6, circles; type 9, squares; and type 15, stars) by various concentrations of rat (open symbols) and human O erythrocytes (filled symbols). The capacity of human O cells to remove type 6 and 9 virion HA was similar. For this reason only symbols of the former are shown.

cells by virions of subgroup II was not affected by the presence of either homotypic or heterotypic fibers irrespective of the technique used.

Adsorption of purified virions of serotypes 6, 9, and 15 by human O and rat erythrocytes. The results obtained in the interference experiments suggest a variation between different serotypes with regard to their capacity to interact with human O and rat red cells. In order to compare representative serotypes with regard to this characteristic, adsorption of different amounts of purified virions by various concentrations of erythrocytes were performed. In these preliminary experiments serotypes 2 and 6 gave similar results. Out of these serotype 6 was chosen as a representative of subgroup III, together with the subgroup II members, serotypes 9 and 15, for further studies. The results presented in Fig. 3 are based on three individual experiments in which a preselected constant amount of virions of each serotype was adsorbed by various concentrations of red cells. The slope of adsorption curves for the different serotypes did not differ significantly and indicated the proportional reduction of virions by adsorption with erythrocytes. The length of the shoulder of the adsorption curve was considered to reflect the relative number of virus specific receptors on the erythrocytes. Rat red cells displayed a considerably higher capacity than human erythrocytes to adsorb virions of the serotypes studied. The highest concentration of rat red cells at which, under the experimental conditions used, no reduction in HA titer of serotypes 15, 9, and 6 was noted, were 0.16, 1.3, and 2.7%, respectively. These values should be compared with 10% human O red cells for both types 6, and 9 and 30% for serotype 15.

Effect of RDE on the agglutination of human erythrocytes by adenovirus type 6 *virions*. The effect of receptor destroying enzyme (RDE) was studied since this can elute serotype 9 HA's from human O erythrocytes (17). A fixed amount of red cells was incubated at room temperature for 60 min with serial twofold dilutions (0.2 ml of physiological saline) of standard cholera filtrate (N.V. Philips-Roxane, The Netherlands). 0.2 ml of virus preparations containing 4 HAU/ml was added to each sample. In order to control the activity of RDE, allantoic fluid of influenza A_2 Tokyo 3/67 (kindly provided by Dr. Leo Heller) and preparations of adenovirus types 9 and 10 were included in these experiments (Table III). The agglutination of human O red cells by the three control preparations was affected to a considerable extent by RDE treatment, as could be expected (10, 17, 21), whereas no detectable effect on the hemagglutination by virions of serotype 6 could be noted.

HI tests with different kinds of HA's of adenovirus types belonging to subgroup III. Preparations of serotypes 2, 4, and 6 were chosen for this study since they represent different patterns of occurrence of soluble complete and incomplete HA's (see above).

The relative differences in HI titers obtained in tests with various complete HA's were similar when different hyperimmune sera against one and the same serotype were compared. For this reason and in order to reduce the complexity of Table IV only the results of testing of two sera per serotype are presented. Among the different complete HA's employed, incomplete HA's aggregated by heterotypic antisera and virions gave the highest HI serum titers. The use of spontaneously occurring aggregates of fibers and pentons and unfractionated virus preparations regularly yielded lower serum titers. A 16- and 8-fold difference in HI titers was observed for serotypes 2 and 6, respectively, when the antigens giving the highest HI, titers were compared to unfractionated virus preparations. Serotype 4 demonstrated only a fourfold difference in HI titer when the corresponding preparations were compared.

Discussion. The partial hemagglutination of rat cells by adenoviruses belonging to subgroup III seemed to be caused by the occurrence in unfractionated preparations of a relative excess of incomplete hemagglutinins, *i.e.*, monomers of pentons and fibers compared with the complete HA's. This explanation was based on observations of the complete or almost complete hemagglutination of rat cells by purified virions and soluble complete HA's, which previously have been identified ultrastructurally as polymers of pentons or fibers (4, 5, 9). Furthermore, it was reported (19) that complete hemagglutination can be obtained with unfractionated virus materials after incubation with heterotypic antisera. In this case the incomplete HA's probably are combined by antibodies, thus forming hemagglutinating complexes. In addition the comparatively low number of receptors on rat red cells reacting with serotypes belonging to subgroup III, indicated by the relatively poor adsorption of virions to rat red cells and by the rather effective blocking of their agglutinating activity by isolated fibers, probably play a considerable role for the character of the observed agglutination patterns.

The fact that the agglutination of human O erythrocytes by the subgroup II member, type 15, and subgroup III members, types 1, 2, 4, 5, and 6, presented above, previously has remained undetected probably is due to the very faint activity demonstrable in HA tests with unfractionated material. The bottom patterns obtained with human O red cells generally displayed a more partial nature than those with rat cells. This difference might be explained by the findings (a) that human red cells seem to contain fewer receptors than rat cells judging from the results of adsorption experiments and connected here to (b) that fiber incomplete HA interferes more effectively with the agglutination by virion complete HA of human than of rat red cells. The agglutinability of cells from different donors of human cells varied considerably. This might be due to a variation in concentration of receptors on cells derived from different individuals.

It seems likely that the distal part of adenovirus fibers is responsible for the interaction with receptors on cells and also represent the site of attachment of HI anti-

TABLE III. The Effect of RDE on the Capacity of Human Red Cells to Become Agglutinated by Influenza A_2 and Adenovirus Types 6, 9, and 10.

Influenza A_2 Tokyo 3/67	Crude preparation of adenovirus type 10	Virions of adenovirus type 9	Virions of adenovirus type 6
64[a]	64	32	<4

[a] Titers given represent the reciprocal of the highest dilution of RDE which completely abolished the agglutination of the red cells by 4 units virus HA.

bodies. In view of the type-specific nature of the latter it is of interest that isolated fibers could interfere with the agglutinating activity of not only virions of the same serotype, but also all other serotypes tested, which agglutinated the same kind of cells. No interference with the interaction between subgroup II virions and rats cells could be demonstrated, under the condition used. However, it could not be excluded that in the presence of considerably larger quantities of fibers this kind of hemagglutination might also be affected. The difference in specificity between HI tests and fiber interference tests can be explained in some different ways. One possibility would be that fibers of different serotypes interact with different receptors, in which case the fiber interference phenomenon must be due to steric hindrances established on the red cell membrane. This mechanism most likely is operating in a case when fibers of subgroup III members block the activity of type 9 virions, since only the receptors for the latter type appears to be sensitive to RDE treatment, suggesting qualitative differences. An alternative explanation, which might concern fiber interference phenomena between members of the same subgroup, could be that fibers of the different serotypes combine with the same type of receptor, but that the receptor combining site is restricted to a part of the knob of fibers, which does not carry an antigenic determinant. This situation basically could be similar to that found

TABLE IV. HI Titers Obtained with Different Types of HA's of Adenoviruses Belonging to Subgroup III.

	Hyperimmune sera against					
Type:	2		4		6	
Antigen preparation	Unfractionated material	Pentons	Unfractionated material	Pentons	Unfractionated material	Pentons
Unfractionated material	7[b]	7	12	11	10	10
Virions	10	10	12	12	12	11
Dimers of pentons (types 2 and 6) or dodecons (type 4)	9	9	12	13	11	12
Aggregates of fibers	9	10	—[c]	—	NT[d]	NT
Penton incomplete HA plus anti-adenovirus type 3 dodecon serum[a]	11	11	14	14	13	14
Fiber incomplete HA plus anti-adenovirus type 5 fiber serum[a]	11	12	14	14	12	13

[a] The isolated incomplete HA's were combined by incubation with heterotypic antisera to form hemagglutinating aggregates prior to their mixing with homotypic antisera.

[b] All titers are expressed in $\log_2$ values and obtained with 4 HAU of antigen.

[c] Aggregates of fibers have not been demonstrated for serotype 4.

[d] NT = not tested.

for polio and Coxsackie B virus receptors on HeLa cells (22) and for adenovirus receptors on HeLa and KB cells (23).

Application of rat erythrocytes at room temperature can be recommended for routine hemagglutination with adenoviruses belonging to subgroups II and III. The main disadvantage of using human O red cells is the fact that there is a much greater variation in agglutinability of these cells derived from different donors as compared to rat cells. When the latter cells were used in tests aiming at an evaluation of the optimal type of antigen preparation of subgroup III members, for HI tests, incomplete HA's combined by heterotypic antisera were found to be best. For practical purposes this implies that incubation of the unfractionated virus material with heterotypic antisera as originally suggested by Rosen (19) gives the most sensitive HI system.

Summary. The hemagglutination by adenovirus types 1, 2, 4, 5, 6 (subgroup III), and 9, 15 (subgroup II) was studied. Serotype 9 displayed, as should be expected, a complete agglutination of human red cells. In addition these cells were agglutinated partially by all the remaining serotypes, although a considerable variation in agglutinability of human erythrocytes obtained from different donors was demonstrated. The hemagglutination of these red cells by serotype 9 was RDE sensitive, whereas the agglutination of human cells by the remaining serotypes was resistant to this treatment. The partial agglutination of both human O and rat red cells peculiar to unfractionated preparations of members of subgroup III was suggested to be caused by the presence of a relative excess of incomplete HA's (i.e., fibers and pentons), in unfractionated preparations of these types, which competitively interfered with the complete hemagglutinin. The following observations supported this explanation (a) isolated complete HA's, such as virions, displayed complete agglutination with both kinds of red cells; (b) adsorption experiments demonstrated a larger amount of virus specific receptors on rat red cells reacting with members of subgroup II than with members of subgroup III; and (c) in a model system the presence of fibers belonging to both subgroup II and III competitively inhibited the virion-mediated agglutination of both rat and human cells.

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