A New Serological Technique for Identification of Adenovirus Infections[1] (34905)

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(Introduced by J. F. Enders)

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The regular procedure to identify adenovirus infections includes virus isolation combined with complement fixation (CF) tests for demonstration of group-specific antibodies. In order to reveal the specific serotype responsible for an infection, neutralization or hemagglutination-inhibition (HI) tests have to be employed.

Group-specific antibodies demonstrated by the CF test have been found to indicate an antigenic component present in nonvertex capsomers, hexons (1, 2). Recent studies have shown that also vertex capsomers carry a group-specific antigenic component, which, however, is distinct from that of hexons (3). Antibodies, against the group-specific component of vertex capsomers have been found to be capable of converting penton incomplete hemagglutinin (HA) into a hemagglutinating aggregate [cf. Ref. (4)].

By analogy with neutralizing and HI antibodies one would expect group-specific penton hemagglutinating-enhancing (HE) antibodies to show a lower rate of time-dependent decline in titer than CF antibodies (5). The prevalence of HE antibodies of this kind therefore might represent an indicator of previous experiences of any kind or number of adenovirus infections.

Results are presented of studies of (a) the frequency of occurrence and titer levels in different age groups of HE antibodies reacting with vertex capsomers of pentons of adenovirus types 3 and 11, and (b) the possible practical usefulness of booster responses of these antibodies as an indicator of a recent infection. Penton incomplete HA of type 11 represents the most sensitive indicator for antibodies reacting with vertex capsomers (3). However, in the case of infections with serotype 11 the appearance of HI antibodies might preclude the demonstrability of antibodies against vertex capsomers by HE tests with homotypic penton incomplete HA. For this reason penton incomplete HA of type 3 was also included in the antigen preparation.

Materials and Methods. Sera. 203 sera collected from females representing different age groups were kindly put at our disposal by Dr. A. Svedmyr, the Virus Department, Central Bacteriological Laboratory, Stockholm. This collection of sera had previously been used for analyses of the age distribution of sero-immunity to measles, rubella, and Burkitt cells (6, 7).

Studies were also made of 10 paired sera in which significant antibody rises had been found in adenovirus CF and/or neutralization tests and from the donors of which adenoviruses of different serotypes had been isolated during the routine diagnostic activity at the Virus Department of the National Bacteriological Laboratory, Stockholm, Sweden and 30 sera from the Department of Medical Microbiology, University of Nijmegen, The Netherlands, respectively. The 30 paired sera from Nijmegen were tested in Stockholm under code. The interval between collection of acute phase and convalescent sera was about 2 weeks.

Ten batches of gamma globulin (16.5% solution; AB KABI, Stockholm, Sweden) were also included in the study.

In early experiments sera to be examined

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in HE tests were treated with kaolin and thereafter absorbed with red cells. However it was found that this combined procedure was not required. The routine procedure developed was to test all sera at a dilution of 1:40 or higher, without previous treatment and sera at lower dilutions after absorption with an equal volume of a 10% suspension of green monkey (*Cercopithecus aethiops*) erythrocytes.

Antigen. The prototype strains of adenovirus types 3 and 11 were propagated in heteroploid cell lines, Lu 106 or MAS-A cells, as previously described (8). At an advanced stage of cytopathic degeneration, cells from one Roux bottle (about 10^7 cells) were scraped off with a "rubber policeman" and collected after low speed centrifugation in 5 ml of phosphate buffered saline (PBS), pH 7.2 to 7.4. After three cycles of freezing and thawing, cell debris was removed by low speed centrifugation.

The preparation of incomplete penton HA to be used in HE tests must be completely devoid of any complete HA. In order to achieve this, advantage was taken of previous findings that soluble complete hemagglutinins (dodecons) of adenovirus types 3 (8) and 11 (9) can be removed more readily by absorption with red cells than penton incomplete HA. The virus materials were absorbed repeatedly with packed 10% green monkey erythrocytes until all complete HA had been removed. It should be pointed out that, in the case of type 11, a too extensive absorption with red cells may remove also incomplete HA. Thus caution should be taken only to barely remove all complete HA activity of this kind of material.

Performance of the hemagglutination enhancement (HE) test. As a first step the amount of incomplete HA in the absorbed preparations has to be determined. In the case when no sera of known HE titers are available chess-board titrations have to be performed. Antisera against any heterologous serotype can be employed. Furthermore gamma-globulin preparations can be used for this purpose, since their content of HE antibodies markedly exceeds that of HI antibodies against types 3 and 11 (see Results). The knowledge of the approximate HE titers of gamma globulin in tests with incomplete HA of types 3 and 11 (see Table I) simplifies the selection of dilutions to be used in chess-board titrations. After testing, the preparations of incomplete HA were pooled and diluted to contain 4 units of each antigen/0.025 ml.

In the final test, serial twofold dilutions of sera (0.025 ml) were mixed with an equal volume of the pretested antigen pool in disposable microtiter plates (Flow laboratories Ltd, Irvine, Scotland). After mixing and in-

TABLE I. Adenovirus HI and Penton HE Antibodies Against Serotypes 3 and 11 in 10 Batches of Gamma Globulin.

HE antibodies were determined both with a pool of penton incomplete HA of the two serotypes and with each of them separately.

	HE titers against penton incomplete HA of types:			HI titers against type:	
Batch no.	3 + 11	3	11	3	11
1	4000	4000	8000	40	10
2	4000	2000	4000	40	10
3	4000	2000	8000	40	20
4	8000	2000	8000	40	40
5	4000	2000	8000	40	20
6	8000	2000	8000	40	20
7	4000	4000	8000	40	20
8	8000	4000	8000	40	20
9	4000	2000	8000	40	20
10	4000	2000	8000	40	20

cubation for 1 hr at room temperature, 0.025 ml of an 0.5% suspension of green monkey erythrocytes was added. Readings were taken after the red cells had settled in a 37° incubator. The last well exhibiting a bottom pattern of complete or clearcut partial agglutination was taken to contain one HE unit (HEU) of serum. In exceptional cases, when antibodies are demonstrable only at low serum dilutions, the presence of relatively large amounts of serum cause aggregated cells to slide down the sides of the well. The reading of bottom patterns of this kind can be facilitated by keeping the rack in an inclined position for a while. Red cells of truly negative bottom patterns slide readily along the wall of the well in a drop-like fashion.

Other serological tests. Sera from the Department of Medical Microbiology, Nijmegen, The Netherlands, were tested in complement fixation (CF) and in some cases in neutralization tests (NT) by use of techniques described previously (10). CF antibodies in sera from the Department of Virology, National Bacteriological Laboratory, Stockholm, were determined by use of an adenovirus type 6 preparation as antigen. A microtiter equipment was used. Two units of antigen and complement and 6 amboceptor units were employed. Hemagglutination-inhibition (HI) tests were performed as described in an earlier publication (11).

Results. The occurrence of penton HE antibodies in different age groups. Sera taken from females representing different age groups ranging from 2 to 40 years or higher were tested for their content of HE antibodies demonstrable by the pooled antigen of penton incomplete HA of types 3 and 11. The results are summarized in Fig. 1.

As should be expected (12) a large percentage of children had passed through their first adenovirus infection already at the age of 2 years. It seems that once this has occurred penton HE antibodies remain at a titer of 40 or higher throughout life. Only rarely were titers as low as 20 encountered. At the age of 15 years or older 95% or more of all individuals were seropositive. The average HE titer of positive sera in these age groups was about 200.

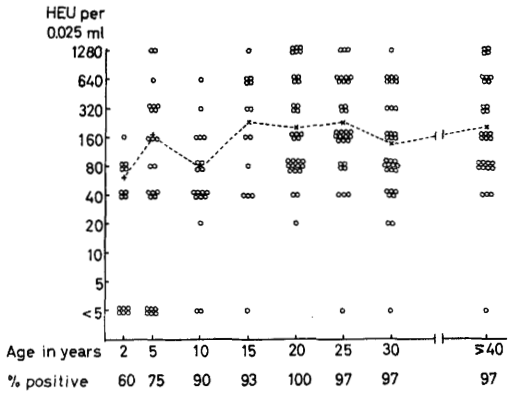

FIG. 1. HE antibodies reacting with penton incomplete HA of types 3 and 11 in sera from females representing different age groups: Percentage of positive sera is indicated below the abscissa; (---), mean antibody titers of positive sera.

Titers of penton HE antibodies in normal gamma globulin. Ten batches of normal gamma globulin were tested for penton HE antibodies and also HI antibodies against types 3 and 11. HE titers of the order of magnitude of 4000 were found in tests with the pooled antigen of penton incomplete HAs of types 3 and 11 (Table I). Titers obtained with type 3 penton incomplete HA were somewhat lower, 2000 to 4000; whereas those found with type 11 incomplete HA were slightly higher, about 8000. HI antibody titers against soluble components of types 3 and 11 were about 40 to 20, respectively. The latter results agree with data, which are already available (13). It should be noted that the HE titers markedly exceed those of HI antibodies. Gamma globulin therefore can be usefully employed for demonstration of incomplete HA.

Analysis of the potential usefulness of penton HE tests for serological diagnosis of adenovirus infections. 30 paired sera from the Department of Medical Microbiology, Nijmegen, The Netherlands, were analyzed in Stockholm under code for penton HE antibodies. Cases representing infections with various serotypes and of different ages were included. All patients infected wih serotypes 1, 3, 4, 7, and 21 displayed acute respiratory disease. Infections with serotype 8 were associated with keratoconjunctivitis. The results

TABLE II. Penton HE Titers in Paired Sera from Cases of Adenovirus Infections Previously Identified by CF and/or Neutralization Tests and by Virus Isolation at the Department of Medical Microbiology, Nijmegen, The Netherlands.

Cases infected with serotype 1, 3, 4, 7, and 21 displayed acute respiratory disease; whereas infections with type 8 were associated with keratoconjunctivitis.

Patient	No.[a]	Age (years)	Sex[b]	Adenovirus isolation (type)	Serum titers		
					NT[c]	CF	HE
1	I	0.75	F	1	<4	<5	<10
	II				16	<5	20
2	I	18	M	1		<5	2560
	II					10	10,240
3	I	16	F	3		10	320
	II					40	2560
4	I	19	M	3		<5	160
	II					40	640
5	I	4	F	3		5	2560
	II					40	40,960
6	I	18	M	4	<4	<5	640
	II				≥4096	20	5120
7	I	20	M	4	<4	<5	160
	II				64	40	2560
8	I	19	M	7		<5	20
	II					640	10,240
9	I	19	M	7		<5	320
	II					40	5120
10	I	74	M	8	8	<5	1280
	II				64	5	1280
11	I	46	F	8	<8	<5	1280
	II				8	20	5120
12	I	37	F	8	<8	20	320
	II				64	40	1280
13	I	20	M	21		<5	1280
	II					40	2560
14	I	19	M	21		<5	640
	II					≥80	20,480
15	I	20	M	21		<5	40
	II					20	5120

[a] Acute phase (I) and convalescent (II) sera were collected at a time interval of about 2 weeks.
[b] F = female; M = male.
[c] Only determined in selected pairs of sera.

of testings of 15 of the 30 paired sera are summarized in Table II.

Most pairs of sera included in Table II and *all* 15 pairs not included in Table II displayed distinct antibody rises both in the conventional CF test and in the penton HE test. Sera from some patients included in Table II deserve special comments. Patient No. 1, a girl aged 9 months, apparently had contracted her first adenovirus infection, which was demonstrable by virus isolation and also by antibody rises in NT and HE tests, but *not* in CF tests. In patient No. 10, a case of keratoconjunctivitis caused by type 8, no rise in HE antibodies was demonstrable. An 8-fold increase in NT antibodies and

TABLE III. Penton HE Titers in Paired Sera from Cases of Adenovirus Infections, Previously Identified by CF Tests and by Virus Isolation at the Department of Virology, National Bacteriological Laboratory, Stockholm, Sweden.

Symptoms of cases 2 and 3 included fever and diarrhea. Remaining cases displayed acute respiratory disease.

Patient	No.[a]	Age (years)	Sex[b]	Adenovirus isolation (type)	Serum titers	
					CF	HE
1	I	21	F	3	10	320
	II				80	5120
2	I	8	M	7	<5	<10
	II				20	2560
3	I	7	F	7	<5	640
	II				80	20,480
4	I	19	F	7	10	1280
	II				20	20,480
5	I	13	F	7	10	320
	II				80	10,240
6	I	27	M	7	10	320
	II				80	20,480
7	I	22	M	7	<5	20
	II				40	1280
8	I	19	M	7	10	160
	II				80	2560
9	I	17	M	7	20	20
	II				40	5120
10	I	22	M	7	<5	80
	II				10	1280

[a,b] See corresponding footnotes of Table II.

the appearance of barely detectable amounts CF antibody were found in this case. In patient No. 12 the rise in HE antibody titers was 4-fold; whereas only a 2-fold rise (not significant) in CF antibody titers was found. Patient No. 13 displayed the reversed situation of relative rises in HE and CF antibody titers.

Out of 10 paired sera made available from the Department of Virology, National Bacteriological Laboratory in Stockholm, 9 pairs had been collected from patients infected with adenovirus type 7. Two of these patients had enteric symptoms (Nos. 2 and 3); whereas the remaining seven patients were cases of acute respiratory disease of varying degrees of severity. The tenth case displayed respiratory symptoms caused by an infection with type 3. In all 10 cases, 16-fold or greater rises in penton HE antibody titers were found (Table III). Rises in CF antibody titers were less pronounced and in two cases, patients 4 and 9, only a twofold increase in titer was recorded.

Discussion. The general pattern of the accumulated appearance of group-specific penton HE antibodies in different age groups found in the present study can be described as follows. About 75% of all children had experienced their first adenovirus infection before the age of 5 years. This is in agreement with results of previous investigations in which NT antibodies against some different selected serotypes were determined (12). After the primary adenovirus infection HE antibodies of a titer of 40 (in a few cases 20) or higher seem to be retained throughout life. The titer of penton HE antibodies is markedly boostered by a renewed adenovirus infection. Judging from the limited experi-

ence derived from the present investigations it seems that tests for content of penton HE antibodies may have an application in diagnostic adenovirus serology as an alternative to the conventionally employed CF test. This raises the following question. Does the penton HE test offer any advantages over the CF test based on group-specific antihexon antibodies?

Concerning the practical aspects of setting up the HE test it can be mentioned that the procedure for preparing penton incomplete HA is relatively simple. The amount of penton incomplete HA in erythrocyte absorbed material is readily determined in chess-board titrations with gamma globulin. Also it can be mentioned that the content of penton HE antibodies in gamma globulin might be used for standardization of this kind of product. In the present study a pool of penton incomplete HA was used for demonstration of HE antibodies. This pool included type 11, which represents the best indicator for penton HE antibodies (3), and type 3. The latter was used to ensure that possible infections with type 11 should also be detected by the antigen. It might be noted, however, that from the point of view of the epidemiology of adenovirus infections (5), this serotype seems to be of minor importance. Additional characteristics of preparations of incomplete HA to be mentioned, are that they usually contain large amounts of antigen, which allows the use of only small amounts of stock material for a large number of tests, and furthermore that they are readily stored in the frozen state, or even for weeks at 4°C, without any loss of activity (Norrby; unpublished data).

It seems most rational to test sera at an initial dilution of 1:40, since patients with previous experience of adenovirus infections already in their acute phase serum specimen have HE titers of this order of magnitude or higher. Because of this only small amounts of sera are needed and furthermore they do not require any special treatment prior to testing. Sera from cases in which no HE antibodies can be demonstrated at a dilution of 1:40, should be retested at a 1:4 dilution after absorption of serum with red cells. In our experience nonspecific inhibitors have not caused any disturbancy and distinct bottom patterns were readily obtained. From the technical point of view it seems therefore that the HE test offers some advantages over the CF test.

In very young children it has been observed that adenovirus infections may give rise to a significant increase in neutralizing antibodies; whereas the CF antibody response may be poor or even not demonstrable (14). It is possible that the HE test may turn out to be particularly useful in these cases. Patient No. 1 in Table II may in point of fact represent an example of this. Furthermore it has been described (15) that adenovirus infections in adults occasionally may be associated with a delayed increase or, in a few cases, no increase in CF antibody titers. It remains to be seen whether the penton HE test in these cases is a better indicator of a recent infection than the CF test.

The group-specific HE antigenic component of vertex capsomers has been found not be confined to human serotypes of adenoviruses only. Both simian, dog, and bovine adenoviruses seem to carry smaller or larger amounts of this antigen (Norrby; to be published). The serological technique described above may therefore be applicable also to analyses of adenovirus infections in animals.

Summary. Titers of antibodies against a group-specific antigenic component of vertex capsomers of human adenoviruses have been determined in sera from healthy and adenovirus-infected individuals. For this purpose a hemagglutination-enhancement (HE) test based on the capacity of antibodies to convert penton incomplete hemagglutinins of types 3 and 11 into hemagglutinating aggregates, was employed.

Penton HE antibodies had been acquired in about 75% of all individuals before the age of 5 years and the percentage approached 100 at the age of 15 years. After the primary adenovirus infection, penton HE antibodies appeared to remain at a titer of 40 or higher. Renewed adenovirus infections caused booster increases of the base line HE titers. These rises of antibody titers can be practi-

cally employed for serological diagnosis of adenovirus infections. The relative usefulness of the penton HE test as compared to the group-specific complement fixation test is discussed.

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