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Vero Microcultures for Adenovirus Neutralization Tests

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A microculture neutralization test is described for measuring specific antibody levels to the 35 human adenovirus serotypes in Vero cells. The test is read at 5 days by macroscopic observation after staining the uninfected cells with crystal violet. The test is performed with a minimum of manipulations and gives serum titers comparable with those obtained in tube macrocultures of monkey kidney, human embryonic kidney, and Vero cells. The Vero microculture neutralization test measures inhibition of adenovirus toxicity, although selected human adenoviruses serially subpassaged in Vero cells were shown to successfully adapt and replicate in the absence of detectable helper viruses.

Diagnostic adenovirus serum neutralization (SN) tests are often performed in primary rhesus monkey kidney (MK) cells because the test can be read in 3 days and then discarded without additional manipulations. The 3-day test, originated by Rowe et al. in 1958 (21) and later described in more detail (26), is based on neutralization of the cellular toxicity of the penton antigen and/or vertex capsomere (10, 14-17, 29, 30), because human adenoviruses do not replicate in primary simian cells in the absence of a helper virus such as latent simian virus 40 (SV40) (1, 2, 7, 13, 18, 22, 23). SN tests in which viral infectivity is neutralized are conducted in human epithelial cell cultures (e.g., FL, HEp-2, KB, HeLa, HEK, pHAm) and can only be read after 7 to 14 days (19). Thus, the MK test has generally been preferred over the longer SN tests because the time for holding the test is shorter, the maintenance steps are fewer, and the cytopathology (CPE) is much easier to read.

Currently, however, the increasing scarcity of rhesus monkeys calls for diligent measures both to conserve the species in its native India and to allow time for monkey farms in this country to breed adequate numbers of juveniles for future use. The present study was thus carried out to evaluate an adenovirus SN test which required relatively short incubation times in a culture system not dependent upon a supply of rhesus monkey.

MATERIALS AND METHODS

Cell lines. MK cells were obtained weekly from the Tissue Culture Unit, Center for Disease Control, Atlanta, Ga. Vero and BS-C-1 African green monkey kidney cell lines were initially obtained from HEM Research Inc., Rockville, Md. Primary human embryonic kidney (HEK) cells were obtained as needed from HEM Research or from Microbiological Associates, Bethesda, Md. Other cell cultures were taken from our own stocks. All cultures were maintained as previously described (9).

When needed for microcultures, cells in a 32-ounce (ca. 960-ml) prescription bottle (120-cm^2 surface) were trypsinized; suspended in 10 ml of growth medium (GM) (Eagle minimal essential medium with 10% fetal calf serum, 0.14% bicarbonate, and 50 μg of chlortetracycline [Aureomycin] per ml); aspirated thoroughly to break up clumps; and diluted with another 10 ml of GM. A small volume of the suspension was mixed with an equal volume of 0.1% crystal violet in 0.1 M citric acid for cell counting with a Spencer-Neubauer hemacytometer (American Optical Co.).

Viruses and antisera. Prototype strains of human adenovirus (AV) serotypes 1 through 35 and their subsequent working stocks (proven free of mycoplasmas, adenovirus-associated viruses, and other contaminants) have been described in previous reports (9, 11, 25). Reference equine antisera to serotypes AV 1 through 35 have similarly been described (9, 11, 25). Virus (infectivity or toxicity) titrations and SN tests in MK, HEK, and Vero tube cultures were carried out as described (9, 11, 21, 26).

Microcultures. Virus titrations and SN tests were done in sterile, 96-well, flat-bottomed, styrene plates (M-220-29-AR or 1-220-29-TR, Cooke Laboratory Products, Alexandria, Va.). Dilution series were made in the plates either manually with standard microtiter equipment or with an Automatic Diluter (model 222-20-PU, Cooke) with 0.050-ml loops (222-23). Viruses, antisera, and cells were always diluted in GM for use in the microtest. Plates were sealed with pressure-sensitive film (3044, Falcon Plastics, Oxnard, Calif.) and incubated for 5 days at 35.5°C under 5% CO_2 and 80% humidity. No additional maintenance steps were required. After the tape was removed and the culture fluid discarded, the plates were immersed in a fixative/stain solution for 20 min, rinsed briefly in water, and inverted on a towel to air dry. The stain solution consisted of 1.46 g of crystal violet (Eastman, 96% dye content) in 50 ml of 95% ethanol, 300 ml of 37% (wt/wt) formaldehyde (Fisher, reagent), 650 ml of distilled water, and 1,000 ml of 0.01 M phosphate-buffered

saline, pH 7.2 (1.096 g of Na_2HPO_4, 0.315 g of $NaH_2PO_4 \cdot H_2O$, 8.5 g of NaCl, made to 1,000 ml with distilled water). The stain solution thus had final concentrations of 5.55% formaldehyde and 0.07% crystal violet, and a final pH of about 6.5.

RESULTS

Evaluation of cells. Many cell types were evaluated in preliminary investigations for use in the microculture neutralization test. A usable cell type had to form confluent monolayers in the plate wells, support the growth of human adenoviruses or indicate penton toxicity, and be readable after a reasonably short incubation period either on the basis of CPE or staining of uninfected cells. For various reasons, BS-C-1, HEp-2, KB, Chang Conjunctiva, secondary HEK, and RM-6 cells were unacceptable for the test.

The Vero cell line, however, appeared acceptable because it is a continuous cell line which grows confluently and in which adenoviruses produce CPE throughout the cell sheet. As determined by cell counts in the 0.05% acidic crystal violet stain, initial cell concentrations of 200,000 cells/ml gave confluent growth in the plates in 3 days, and 175,000 cells/ml produced confluent growth in 5 days. At the latter concentration, one confluent monolayer of Vero cells in a 32-ounce (ca. 960-ml) bottle, when trypsinized, counted, and diluted, provided cells for 16 to 20 plates. Although tests with the "slower-growing" adenoviruses, e.g., types 12, 13, 14, 16, 18, and 29, tended to be more easily read at 7 days than at 5, the test was constructed as a 5-day test.

Microculture virus titrations. Virus titrations were carried out in replicas of six in the microtiter plates. With sterile droppers, 0.10 ml of the undiluted virus stock was added to well 1, and 0.05 ml of GM was added to wells 2 through 12 of each of the 6 rows. The virus was then serially diluted with flame-sterilized, 0.05-ml loops, and an additional 0.05 ml of GM was added to each titration well. The two rows of cell control wells contained 0.10 ml of GM only. To the entire plate, 0.05 ml of the Vero cell suspension (175,000 cells/ml) was added per well. The plate was gently tapped for mixing, sealed with sterile film, incubated for 5 days at 35.5°C, and stained with 0.07% crystal violet as described above.

End points were read in the wells which showed ≤25% of the stained area (i.e., uninfected cells) present in the uninoculated cell control wells. The virus titer in Vero microcultures was thus defined as the dilution factor of the highest dilution of virus showing 0 to 1+ staining (4+ to 3+ CPE) in 5 days; this dilution was considered 1 U of virus per 0.05 ml. A typical microtitration is shown for one serotype in Fig. 1A. The working stocks of all 35 serotypes were titrated in this manner. The results are compared in Table 1 with the titers measured in HEK, MK, and Vero tube cultures.

Microculture SN titrations. SN titrations were carried out in replicas of six, and the serum controls in duplicate, in microtiter plates. With sterile droppers, 0.10 ml of untreated antiserum (usually at a 1:10 starting dilution) was added to well 1, and 0.05 ml of GM was added to wells 2 through 12 of each of the 8 rows. The serum was serially diluted with flame-sterilized, 0.05-ml loops. To each of the 12 wells in the 6 serum titration rows was added 0.05 ml of a virus suspension containing 2 U of virus per 0.05 ml (i.e., if a 1:64 dilution of virus = 1 U of virus per 0.05 ml, then a 1:32 dilution of virus = 2 U/0.05 ml). To each of the wells in the serum control rows was added 0.05 ml of GM. The plate was gently agitated for mixing and was left for 1 h at 23°C for virus-antibody incubation. After 0.05 ml of Vero cell suspension was added to each well, the test was mixed, sealed, incubated, and stained as above.

The virus back-titration was carried out in a separate plate to determine the actual dose of virus present in the SN test. With sterile droppers, 0.10 ml of the virus suspension used in the SN test was added to well 1, and 0.05 ml of GM was added to wells 2 through 6 in each of 6 rows. The working dilution of virus in well 1 (the virus control) was then serially diluted with 0.05-ml loops, and an additional 0.05 ml of GM was dropped in each back-titration well. The two rows of cell control wells contained 0.10 ml of GM only. The plate was shaken and left for 1 h to incubate as in the SN test. To each well in the half-plate was added 0.05 ml of the Vero suspension, and the test was incubated as above.

Titration end points in the SN test and back-titration plate were read as above. The SN titer of a serum was thus defined as the reciprocal of the highest dilution of serum inhibiting 2 U of virus for 5 days. A proper virus dose was evident in the back-titration by 0 staining in well 1 (virus control), 0 to 1+ staining in well 2, 3+ to 4+ staining in well 3, and 4+ staining thereafter. A representative SN test (Fig. 1B) and back-titration (Fig. 1C) is shown.

Reference antisera to all 35 adenovirus serotypes were similarly tested against their homologous virus. The SN titers are presented in Table 1. The SN titers of the antisera as previously determined in MK tube cultures (11) and as determined in HEK and Vero tube cultures in this study are also listed in Table 1. It is apparent that although the titers of the viruses vary widely from one system to another depend-

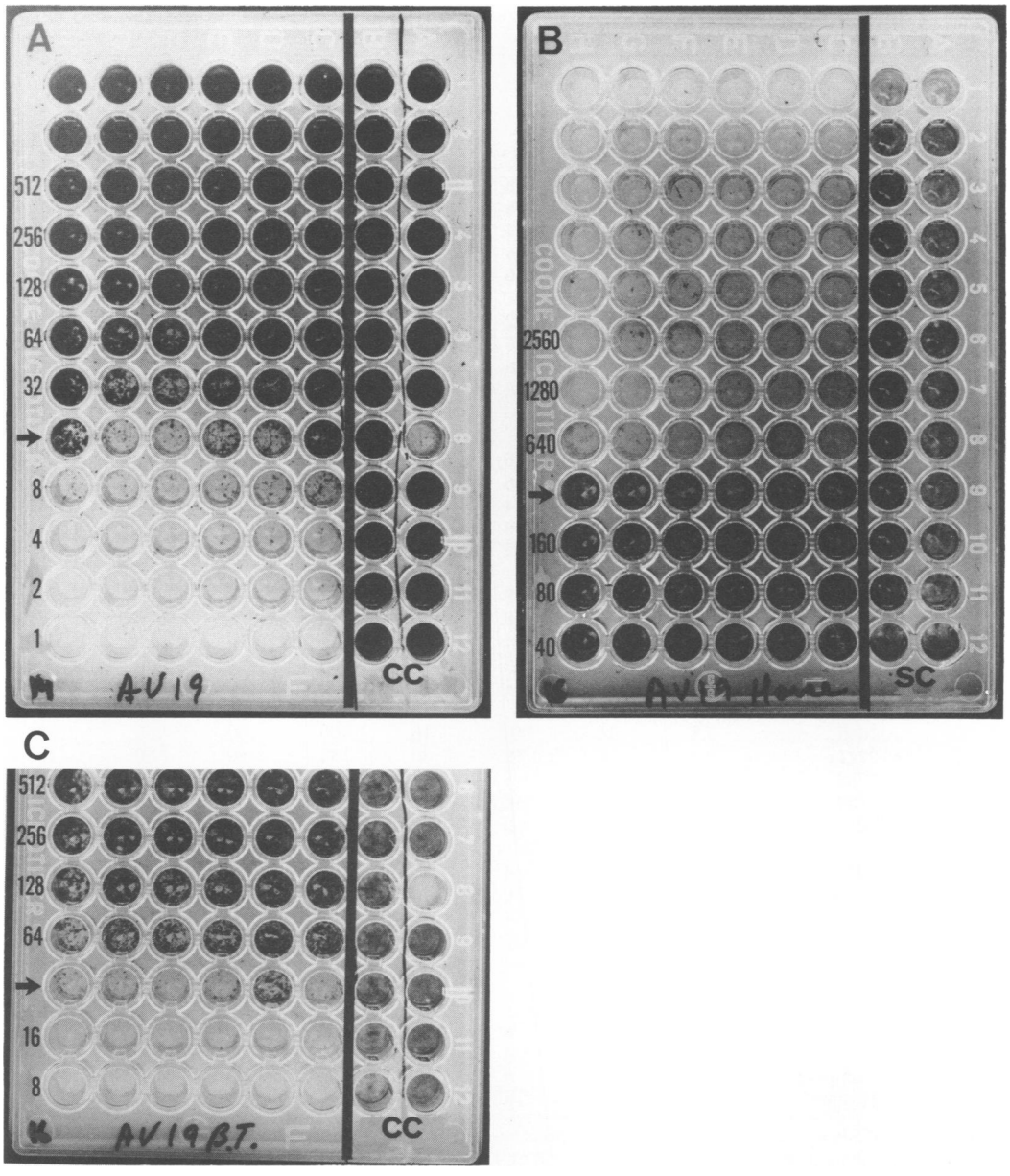

FIG. 1. *Vero microculture tests for serotype AV 19. The virus titration (A) was performed in replicas of six from undiluted through 1:2,048; 24 cell control (cc) wells with the Vero cell suspension as added to the titration are on the right. The SN test (B) with AV 19 reference equine antiserum was performed in replicas of six from a dilution of 1:40 onward; duplicate serum controls diluted at the same time are shown on the right. The back-titration (C) of the virus suspension used in (B) begins with the working dilution of virus (1:8), containing the 2 U of virus, and extends for half of a plate; cell controls again are shown on the right. The arrow in the dilution column of each panel indicates the end point dilution (titer) as defined in the text.*

ing upon the titration series, infectivity versus toxicity, and the definition of the end point in each system, the SN titers are not obviously dependent on the system and thus truly reflect serum antibody levels.

Many of the equine sera exhibited toxicity in the 1:10 dilution or in the 1:10 and 1:20 dilutions, but these levels of toxicity did not interfere with reading the SN end points. Serum toxicity was not removable by heating at 56°C for 30 min

TABLE 1. *Comparison of virus titers and homologous SN titers for 35 human adenovirus serotypes and in various host systems*

Type	Prototype strain	Virus titer[a]				SN titer[b]			
		Tube culture			Microculture (Vero)	Tube culture			Microculture (Vero)
		HEK	MK	Vero		HEK	MK	Vero	
1	Adenoid-71	5.3	64	8	32	160	160	160	640
2	Adenoid-6	4.5	128	64	128	160	320	320	640
3	G.B.	6.3	32	4	32	320	160	80	640
4	RI-67	4.3	64	64	64	640	320	320	320
5	Adenoid-75	4.3	256	256	256	320	320	640	1,280
6	Tonsil-99	3.7	32	32	64	640	320	160	320
7[c]	S-1058	4.5	32	16	32	320	320	320	640
8	Trim	3.5	4	8	4	80	160	160	160
9	Hicks	3.6	16	8	16	160	160	160	160
10	J.J.	4.5	32	16	16	160	160	160	80
11	Slobitski	5.6	16	16	8	160	160	160	160
12	Huie	5.2	8	16	16	160	160	160	320
13	A.A.	1.5	8	4	4	160	160	160	320
14	deWit	6.3	8	8	8	160	160	80	320
15	305	3.8	4	2	16	80	80	160	320
16	Ch. 79	1.5	8	8	8	80	160	160	80
17	Ch. 22	3.5	16	8	8	160	320	320	80
18	D.C.	3.5	4	4	8	160	160	80	160
19	587	5.3	64	32	16	320	320	320	320
20	931	5.8	32	4	8	160	160	160	160
21	1645	5.8	16	16	16	160	160	160	160
22	2711	3.8	16	16	32	80	80	80	160
23	2732	3.6	8	8	8	640	640	320	640
24	3153	3.4	16	16	16	640	320	320	320
25	BP-1	2.6	8	2	2	320	160	320	320
26	BP-2	3.4	8	4	8	160	80	80	80
27	BP-4	4.1	64	16	16	160	160	160	160
28	BP-5	2.5	4	4	4	640	160	160	640
29	BP-6	1.5	8	16	16	640	80	320	640
30	BP-7	2.3	8	4	8	160	80	160	80
31	1315	2.5	4	4	4	640	640	640	1,280
32	H.H.	4.5	4	8	8	160	160	160	160
33	D.J.	1.5	16	4	16	160	160	160	160
34	Compton[d]	5.5	8	4	4	160	160	160	160
35	Holden[d]	5.2	4	4	4	640	640	640	640

[a] Listed for HEK as $\log_{10}$ mean tissue culture infective dose per 0.1 ml at 7 days, for MK as the dilution factor of the highest dilution of virus showing 2+ CPE in 3 or 4 days, and for Vero as the dilution factor of the highest dilution of virus showing 2+ CPE (= 1 U) in 5 days (tubes) or 0 to 1+ staining (= 1 U) in 5 days (microculture).

[b] SN titers are listed as dilution factors of the highest dilution of antiserum: in HEK, inhibiting 30 to 70 mean tissue culture infective doses of virus for 7 days; in MK, causing a 2+ reduction in CPE from that observed in the working dilution (virus control) in 3 or 4 days; and in Vero, inhibiting 2 U of virus for 5 days.

[c] Following the recommendation of Wigand (31), S-1058 (formerly the prototype of AV "7a") should replace the Gomen strain as the prototype of AV 7.

[d] Candidate types (see references 9 and 25).

(and in fact was usually more pronounced after heat treatment) and was not affected by freeze-thaw cycles. We have not observed toxicity in the untreated human sera we have tested for various adenovirus antibodies.

The Vero microculture SN test for adenoviruses was highly reproducible if normal care was taken to ensure that the loops made adequate contact with the medium in the flat-bottomed wells and that the amount of etching of the well

bottom with the loops was minimal. For these reasons it is preferable to use 0.05 ml-volumes for diluting both viruses and antisera and the automatic diluting machine whose loops do not touch the well bottoms. Antisera were tested two to five times during the course of the investigation with different lots of Vero cells, and little variation in SN titers was noted.

Replication of human adenoviruses in Vero cells. The Vero microtest for human adenoviruses was designed to measure either infectivity or toxicity, since either or both may occur. Because Vero cells are of simian origin, albeit a continuous fibroblast cell line, we assumed that the human adenoviruses would not replicate in the absence of a helper virus. Hence we used the same twofold dilution scheme in the virus titration that is used for titrating the virus in primary MK cells. The titers obtained were, in fact, quite similar to the virus titer in MK for each stock tested (Table 1).

However, we felt that the issue of infectivity versus toxicity should be further studied. Accordingly, we selected five serotypes (AV 1, 2, 3, 5, and 7), which had not been passaged in any simian-derived tissue culture, and sequentially passaged them through Vero cells to determine whether virus replication occurred. They were passaged for 6 consecutive weeks, using 0.1-ml inoculum in 0.9 ml of maintenance medium for each passage. The inoculum was not adsorbed to the cell sheet. If the CPE was read as 4+ (that is, 100% of the cell monolayer visibly affected) before the 7-day passage period was over, the tubes were frozen until the next passage. The passages were made each week regardless of when the tubes were frozen or of the degree of CPE at the end of 7 days. Passages four and six were then examined for adventitious agents both serologically and by electron microscopy, and each passage was titrated to infectivity end points in HEK cells.

Simian viruses (viz, SV5, SV13, SV15, SV40, SV41) were not detected with hemagglutination-inhibition or SN tests, and only adenovirus was seen by electron microscopy of supernatant passage fluids. The uninoculated Vero cell control cultures, passaged in parallel with the adenoviruses, were consistently free of any detectable agent. The sixth-passage Vero cultures of the adenoviruses and cell control were blind coded and correctly identified with our routine diagnostic tests (11). Infectivity titers in HEK for the original stocks and fourth and sixth Vero passages are shown in Table 2. Comparison of the observed titer with the expected nonreplication titer (last column) clearly shows that virus replication did occur for all five serotypes tested.

Furthermore, graphic presentation of the infectivity titers of each passage, such as for serotypes AV 1, 5, and 7 (Fig. 2), indicates that adaptation to Vero cells occurred between passages two and four. For AV 1, the titers of passages one, two, and three are slightly lower than the titers expected by simple dilution of the inoculum, suggesting that dilution plus a small loss of virions from environmental factors accounted for these titers. The fourth passage contained newly adapted virus which peaked in infectivity in the fifth passage. For AV 5, the titer of the second Vero passage was 0.6 log U higher than the titer expected by dilution, that of the third passage was 0.8 log U higher, and that of the fourth was 3.5 log U higher, suggesting that some adaptation and replication occurred in the second passage but that most occurred in the fourth. For serotype AV 7, virus replication occurred in the third passage (0.5 log U higher than titer expected by dilution) but principally in the fourth (2.8 log U higher). In no instance did adenovirus replication appear to occur in the first Vero passage.

TABLE 2. *Replication of five common human adenoviruses in Vero cells*

Virus type	Passage history[b]	Infectivity titers in HEK[a]		
		7 days	14 days	Expected 14-day titer if no replication occurred[c]
1	$H_5KB_{10}HEp_1$	5.3	6.8	
1	$H_5KB_{10}HEp_1Vero_4$	4.3	5.7	2.8
1	$H_5KB_{10}HEp_1Vero_6$	5.5	7.7	0.8
2	$xH_{15}KB_{12}HEp_2$	4.5	6.2	
2	$xH_{15}KB_{12}HEp_2Vero_4$	3.5	4.7	2.2
2	$xH_{15}KB_{12}HEp_2Vero_6$	4.5	5.5	0.2
3	$H_7KB_4HEp_2$	6.3	7.5	
3	$H_7KB_4HEp_2Vero_4$	5.8	7.5	3.5
3	$H_7KB_4HEp_2Vero_6$	6.1	7.2	1.5
5	$H_4KB_{13}HEp_2$	4.3	5.8	
5	$H_4KB_{13}HEp_2Vero_4$	3.5	5.3	1.8
5	$H_4KB_{13}HEp_2Vero_6$	5.1	7.1	<0.1
7	$xH_1KB_7HEp_2$	4.5	5.7	
7	$xH_1KB_7HEp_2Vero_4$	3.1	4.5	1.7
7	$xH_1KB_7HEp_1Vero_6$	4.3	5.7	<0.1

[a] HEK titers are listed as $\log_{10}$ mean tissue culture infective doses per 0.1 ml. For each type, titrations were performed on a single day in a single lot of HEK to avoid lot-related variations in virus sensitivity.

[b] H, HeLa cervical carcinoma cell line; KB, KB nasopharyngeal epidermoid carcinoma cell line; HEp, HEp-2 laryngeal epidermoid carcinoma cell line; x, prior passage history documented in reference 11.

[c] Assuming no destruction of infectious virions by the 1:10 dilution of inoculum for each passage, by the 3 to 7 days of incubation at 35°C, or by the single freeze-thaw cycle at −70°C between passages.

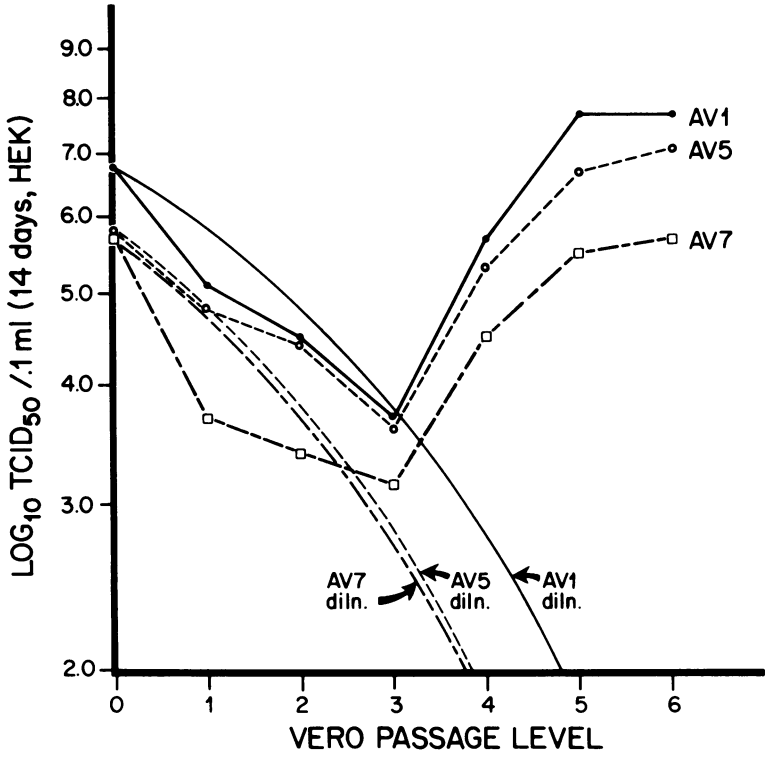

FIG. 2. *Adaptation of serotypes AV 1, 5, and 7 to Vero cells. Graphs show the 14-day infectivity titer per 0.1 ml in HEK cells at each consecutive passage and are compared to the theoretical titer (marked with arrows) expected from dilution only (that is, if no virus replication occurred).*

DISCUSSION

A microculture test system which uses Vero cells is described for human adenoviruses and compared to commonly accepted tests. The development of this test was necessitated by the growing scarcity of rhesus monkeys, which were a principal source of cells for adenovirus SN tests.

Other microculture systems have been used for adenoviruses, but most have one or more disadvantages in that they require either additional manipulations, more time for final results, cells that are not always available, or the individual reading of CPE in each well of the plate. Selected AV serotypes (2, 3, 4, 7, and unknown) have been grown in HEp or HeLa microcultures which were subsequently "read" for CPE (4, 20, 27). AV types 1 through 5, 7, and 10 have been titrated and specifically neutralized in microcultures with human fetal diploid kidney cells which were read for CPE at 4 days (24). In the ongoing Virus Watch Study, AV 1 through 5 and 7 were neutralized in HeLa microcultures, which were read by crystal violet staining at 3 or more days (3). In previous trials, we had titrated AV serotypes 1 through 31 in HEK microcultures, but the cells forming the monolayer were predominantly secondary HEK cells (more fibroblast than epithelial cells), and the CPE was difficult to read. Microculture SN tests based upon these virus titers were nonetheless performed, giving SN titers comparable to those obtained in MK cells (11). However, the test proved to be unsatisfactory because it required 5 to 7 days of incubation; the monolayers were not uniform in the plate, the cells were not consistent from one lot to another, and each well had to be read microscopically for CPE (J.C. Hierholzer, unpublished data). Similarly, AV 1 through 31 have been titrated and neutralized in microsystems utilizing secondary MK and secondary African green monkey kidney cells; the tests were read for CPE after 5 days (5). Avian adenoviruses have been titrated and neutralized in microtests with chicken kidney cells; titers were read by CPE after 6 days of incubation (8).

Vero cells were unique among the cell types included in this study. Vero is a continuous cell line of heteroploid fibroblast-like cells that can be easily maintained in the laboratory, and therefore should be readily available by subpas-

saging once a week at a 2:1 split ratio. Adenovirus CPE occurs throughout the monolayer, rather than only at the edges, and is readily discernable. The Vero line has been successfully used in microculture in our laboratories and elsewhere for such purposes as a microtest for mumps virus (12). The manipulations for the Vero microtests are kept to a minimum by adding the cell suspension directly to the wells after the virus or antiserum has been serially diluted with microtitration loops, rather than growing the monolayers out first and then adding prediluted reagents to the wells.

Many of the reference equine antisera to AV 1 through 35 were toxic at low dilutions for Vero cells in the microculture system. This toxicity was generally limited to the 1:10 and/or 1:20 serum dilutions in unheated sera, but was occasionally 1 dilution higher in sera which had been heated at 56°C for 30 min. Toxicity has not been observed in the unheated human sera we have tested to this point. Thus, antisera tested by the Vero microtest were not heat inactivated; this was also found to be preferable for sera tested in MK tube cultures (11).

No serum toxicity was observed in tube macrocultures of Vero or HEK cells because, in part, cell monolayers which are already confluent in tubes resist low levels of toxicity more readily than do cells in microculture, which must divide and become confluent in the presence of the toxic serum. But serum toxicity may also result from the fact that the antisera (and therefore the toxic factors) are fourfold more concentrated in the microtest (0.05-ml serum dilution in a total volume of 0.15 ml) than in the tube test (0.1-ml serum dilution in a total of 1.2 ml).

The Vero microsystem can detect either adenovirus toxicity, due to "cell-detaching" effects of the vertex capsomere or intact penton antigen, or infectivity due to replication of complete virions. In this study, infectivity experiments with AV 1, 2, 3, 5, and 7 indicated that virus replication began by the second serial passage in Vero cells. This replication occurred in the absence of detectable helper viruses such as adventitious SV40 or a contaminating adenovirus-associated virus. By the fifth and sixth passages, titers of infectious virus (as measured in HEK cultures) reached peak levels approaching those obtainable after serial passage of stock adenoviruses in HEK cells—which are the most sensitive cells for adenovirus replication. Thus these five AV serotypes readily adapted to Vero cells. This is similar to the adaptation observed for AV types 1 through 7 after extensive subpassaging in primary African green monkey kidney cells (6, 28), the cell type from which the Vero line was derived. The adaptation was also similar to that which we have observed when adenovirus isolates are serially passaged in fibroblast cells of human diploid cell strains such as WI-38, HELF, or MRC-5.

The Vero microtest, however, measures adenovirus toxicity rather than infectivity, because each virus titration and SN test is a dead-end "first" passage of stock viruses grown in human cells (Table 2). Furthermore, the test is held for only 5 days, which precludes the opportunity for adaptation to advance as it did in serial, 7-day passages. The virus toxicity explanation is consistent with the observation that virus titers in Vero cells are similar to those in MK cells (Table 1) in which toxicity alone is measured.

The specificity of antibody titers determined by the Vero microtest has thus far been studied to only a limited extent in human adenovirus disease. Paired acute- and convalescent-phase sera from patients with respiratory or conjunctival illness, associated by virus isolation with AV 3, 4, 7, 8, or 19, were tested against all five viruses by microtest. In all cases a fourfold or greater serological conversion to the isolated virus was demonstrated. Heterologous anamnestic titer rises to related serotypes were frequent between types 3 and 7 but were not encountered between types 8 and 19, in accordance with the general prevalence (AV 3 and 7) or nonprevalence (AV 8 and 19) of these viruses. Heterologous titer rises between nonrelated serotypes were not observed. Although these data are limited, the Vero microtest appears to be as type specific as the SN test in MK cells (also a toxicity-inhibiting test) and as the SN test in human epithelial cells (infectivity-inhibiting test). Indeed, most type-specific antigens of an adenovirion reside in the penton component and parts and aggregates thereof (10, 14–17, 22, 29, 30), so that SN tests measuring inhibition either of toxicity or of infectivity are actually measuring different manifestations of the same components of the virus.

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LITERATURE CITED

1. **Alstein, A. D., and N. N. Dodonova.** 1968. Interaction between human and simian adenoviruses in simian cells: complementation, phenotypic mixing and formation of monkey cell "adapted" virions. Virology **35**:248–254.
2. **Boeye, A., J. L. Melnick, and F. Rapp.** 1966. SV40-adenovirus "hybrids": presence of two genotypes and the requirement of their complementation for viral replication. Virology **28**:56–70.
3. **Fox, J. P., C. E. Hall, and M. K. Cooney.** 1977. The Seattle virus watch. VII. Observations of adenovirus infections. Am. J. Epidemiol. **105**:362–386.
4. **Fuccillo, D. A., L. W. Catalano, Jr., F. L. Moder, D. A. Debus, and J. L. Sever.** 1969. Minicultures of

mammalian cells in a new plastic plate. Appl. Microbiol. **17**:619–622.

5. **Furesz, J., R. E. Armstrong, and F. P. Nagler.** 1972. Adenovirus serum neutralization in micro-tissue cultures of rhesus monkey kidney cells. Can. J. Microbiol. **18**:1603–1606.
6. **Goulet, N. R., P. W. Schriver, O. C. Liu, and J. M. Beiler.** 1965. Isolation and propagation of adenoviruses in African green monkey kidney cultures. J. Infect. Dis. **115**:429–435.
7. **Grayston, J. T., M. A. McCarthy, M. Smith, and C. G. Loosli.** 1958. Adenoviruses. II. The sensitivity of different tissue culture strains of cells to adenoviruses. J. Infect. Dis. **103**:86–92.
8. **Grimes, T. M., and D. J. King.** 1977. Serotyping avian adenoviruses by a microneutralization procedure. Am. J. Vet. Res. **38**:317–321.
9. **Hierholzer, J. C., N. O. Atuk, and J. M. Gwaltney, Jr.** 1975. New human adenovirus isolated from a renal transplant recipient: description and characterization of candidate adenovirus type 34. J. Clin. Microbiol. **1**:366–376.
10. **Hierholzer, J. C., and W. R. Dowdle.** 1970. Immunological basis of the adenovirus 8–9 cross-reaction. J. Virol. **6**:782–787.
11. **Hierholzer, J. C., W. C. Gamble, and W. R. Dowdle.** 1975. Reference equine antisera to 33 human adenovirus types: homologous and heterologous titers. J. Clin. Microbiol. **1**:65–74.
12. **Kenny, M. T., K. L. Albright and R. P. Sanderson.** 1970. Microneutralization test for the determination of mumps antibody in Vero cells. Appl. Microbiol. **20**:371–373.
13. **Malmgren, R. A., A. S. Rabson, P. G. Carney, and F. J. Paul.** 1966. Immunofluorescence of green monkey kidney cells infected with adenovirus 12 and with adenovirus 12 plus simian virus 40. J. Bacteriol. **91**:262–265.
14. **Norrby, E.** 1966. The relationship between the soluble antigens and the virion of adenovirus type 3. II. Identification and characterization of an incomplete hemagglutinin. Virology **30**:608–617.
15. **Norrby, E.** 1968. Identification of soluble components of adenovirus type 11. J. Gen. Virol. **2**:123–133.
16. **Norrby, E.** 1969. The structural and functional diversity of adenovirus capsid components. J. Gen. Virol. **5**:221–236.
17. **Pettersson, U., and Stefan Höglund.** 1969. Structural proteins of adenoviruses. III. Purification and characterization of the adenovirus type 2 penton antigen. Virology **39**:90–106.
18. **Rapp, F., and J. L. Melnick.** 1966. Papovavirus SV40, adenovirus and their hybrids: transformation, complementation, and transcapsidation. Prog. Med. Virol. **8**:349–399.
19. **Rose, H. M.** 1969. Adenoviruses, p. 205–226. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral and rickettsial diseases, 4th ed. American Public Health Association, New York.
20. **Rosenbaum, M. J., R. C. Kory, K. A. Siegesmund, H. J. Pedersen, E. J. Sullivan, and R. O. Peckinpaugh.** 1972. Electron microscope methods for the identification of adenoviruses isolated in micro tissue cultures. Appl. Microbiol. **23**:141–144.
21. **Rowe, W. P., J. W. Hartley, and R. J. Huebner.** 1958. Serotype composition of the adenovirus group. Proc. Soc. Exp. Biol. Med. **97**:465–470.
22. **Schlesinger, R. W.** 1969. Adenoviruses: the nature of the virion and of controlling factors in productive or abortive infection and tumorigenesis. Adv. Virus Res. **14**:1–61.
23. **Schmidt, N. J., H. H. Ho, and E. H. Lennette.** 1965. Comparative sensitivity of human fetal diploid kidney cell strains and monkey kidney cell cultures for isolation of certain human viruses. Am. J. Clin. Pathol. **43**:297–301.
24. **Schmidt, N. J., E. H. Lennette, and C. J. King.** 1966. Neutralizing, hemagglutination-inhibiting, and group complement-fixing antibody responses in human adenovirus infections. J. Immunol. **97**:64–74.
25. **Stalder, H., J. C. Hierholzer, and M. N. Oxman.** 1977. A new human adenovirus (candidate adenovirus type 35) causing fatal disseminated infection in a renal transplant recipient. J. Clin. Microbiol. **6**:257–265.
26. **Stevens, D. A., M. Schaeffer, J. P. Fox, C. D. Brandt, and M. Romano.** 1967. Standardization and certification of reference antigens and antisera for 30 human adenovirus serotypes. Am. J. Epidemiol. **86**:617–633.
27. **Sullivan, E. J., and M. J. Rosenbaum.** 1971. Isolation and identification of adenoviruses in microplates. Appl. Microbiol. **22**:802–804.
28. **Tytell, A. A., H. A. Torop, and F. J. McCarthy.** 1962. Adenovirus plaque formation in grivet monkey kidney cells. Proc. Soc. Exp. Biol. Med. **109**:916–918.
29. **Wadell, G.** 1970. Structural and biological properties of capsid components of human adenoviruses, p. 1–39. Karolinska Institutet, Stockholm.
30. **Wadell, G., and E. Norrby.** 1969. Immunological and other biological characteristics of pentons of human adenoviruses. J. Virol. **4**:671–680.
31. **Wigand, R.** 1976. Does adenovirus subtype 7a exist? Arch. Virol. **50**:335–337.