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Quantitative Colorimetric Microneutralization Assay for Characterization of Adenoviruses

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A microneutralization assay that automates the reading and interpretation of viral infectivity and neutralization data was developed for the characterization of adenoviruses (AV). Virus, serum, and cells were added to 96-well plates, incubated for 7 days, and stained with vital stain. The A_{550} of solubilized dye was read in a plate reader interfaced to a personal computer which analyzed the results. Correlation of A_{550} values with visual observation of cytopathic effect was extremely good ($r = \mid 0.977625 \mid$). Clinical isolates of 17 AV from 11 patients were characterized by colorimetric neutralization assay. Prototype AV titers tested were comparable to those determined by tube methods. Prototype homotypic antiserum titers were comparable to or greater than those determined by standard tube neutralization.

The human adenoviruses (AV) are a group of more than forty serotypes which cause a wide spectrum of diseases, including acute respiratory disease and pneumonia, epidemic keratoconjunctivitis, aseptic meningitis, gastroenteritis, acute hemorrhagic cystitis, and serious fulminating infections in the immunocompromised individual (17). Serotyping of isolates is important for epidemiological and public health reasons, but the multiplicity of serotypes necessitates an extensive battery of identification tests. Since the advent of the AIDS epidemic, the characterization of AV has become even more problematic. Several reports have noted the appearance of many intermediate strains that are neutralized, to various degrees, by sera to more than one serotype (9, 10) and the appearance of seven new serotypes isolated from AIDS patients (9, 16). In order to characterize many isolates, they must be titrated against multiple standardized prototype antisera.

Although it was hoped that serotyping by restriction enzyme analysis would be possible (2), it is now recognized that there are multiple genome types for most serotypes (11, 12). While hemagglutination tests are useful to differentiate isolates into subgenera and characterize the fiber attachment protein, neutralization with type-specific antisera remains essential for classification into serotypes (19). The serotyping method proposed by the Centers for Disease Control and Prevention is a 3-day test with primary rhesus monkey kidney cells (5). As AV are species specific and do not replicate in primary rhesus monkey kidney cells, the method does not measure the neutralization of viral infectivity, but instead it measures a reduction in the cytopathic effect (CPE) induced by viral toxicity (5). This test has the advantage of a short incubation period, but the inconsistent availability and expense of primary rhesus monkey kidney cell tube cultures and the frequent deterioration of those cultures due to contamination with simian viruses make reproducibility of results obtained with this test difficult. Neutralization tests that measure a reduction of viral infectivity in various cell cultures, in both tube and microtiter formats, have been described (2, 3, 5, 7). In all of these tests determination of neutralization and virus titer endpoint is based on the visual or microscopic observation of CPE. Depending on the cell type and format used, this observation can be exacting, time-consuming, and subjective. We describe here a microneutralization assay that combines the sensitivity, conservation of reagents, and ease of handling of a microtiter assay with the speed, simplicity, and objectivity of spectrophotometric analysis. We have used the colorimetric neutralization assay (CMN) (i) to serotype AV isolates from clinical specimens, (ii) for serum titrations to quantitate AV antibody neutralization, and (iii) for screening of type-specific neutralizing AV antibody in seroprevalence studies (1).

Viruses and antisera. Prototype AV serotypes, type-specific antisera, and AV intersecting serum pools were from the collection maintained by the Viral and Rickettsial Disease Laboratory of the California Department of Health Services, Berkeley, Calif. Prototype viruses were originally obtained from the reporting investigators; the Research Reference Reagents Branch, National Institutes of Health; the Respiratory Viral Disease Unit, Centers for Disease Control and Prevention; or the American Type Culture Collection. Rabbit antisera to prototype strains were standardized in cross-neutralization tests against all other serotypes. An intersecting pool scheme (15) using standardized antisera was devised. Stock virus cultures were passaged in human fetal diploid kidney cells, the cells were disrupted by vortexing, and cell-free supernatant fluid was frozen at −70°C. Patient strains were originally isolated from human fetal diploid lung cells by the submitting laboratory and then were referred to this laboratory for characterization. Stocks were made in human fetal diploid kidney cells as described above.

Cell culture. Stock cultures of A549 cells (American Type Culture Collection, Rockville, Md.) were maintained by splitting at a ratio of 1 to 6, once a week, in outgrowth medium consisting of 90% Eagle's minimal essential medium in Hanks' balanced salt solution and 10% fetal calf serum (JR Scientific, Woodland, Calif.) buffered with $NaHCO_3$ and supplemented with 600 μg of glutamine per ml, 200 μg of streptomycin per ml, 200 U of penicillin per ml, and 1 μg of amphotericin B (Fungizone) per ml.

CMN. The assay medium for serum and virus dilutions was the outgrowth medium described above. All virus preparations

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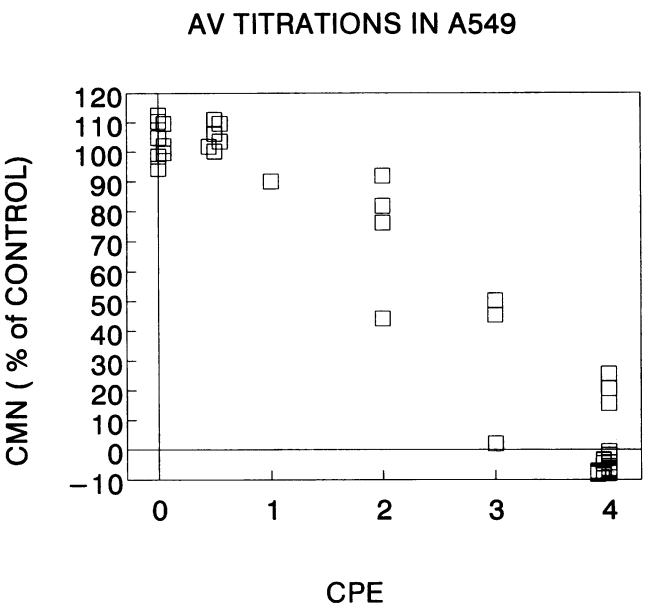

FIG. 1. Correlation of visual determination of CPE with A_{550} values. Absorbance values varied with cell density and so are given as percentages of control values. CPE scoring was done according to conventional methods (14), and the scores were as follows: 1+, 25% of the cells were altered; 2+, 50% of the cells were altered; 3+, 75% of the cells were altered; 4+, 95 to 100% of the cells were altered; and ±, less than 25% of the cells were altered. For graphing purposes a ± score was given a numerical value of 0.5. By linear regression, r was equal to | 0.977625 | . Titrations included prototype AV 2 (AD 6), AV 7 (Gomen), and AV 35 (Holden).

were tested in a preliminary logarithmic serial dilution to determine an optimum working dilution of 100 50% tissue culture infective doses ($TCID_{50}$), with 50 μl per well, by using four replicate wells per dilution. Type-specific antisera were inactivated at 56°C for 30 min and serially diluted twofold in a microtiter plate (Cell Wells; Corning Glass Works, Corning, N.Y.), with 50 μl per well and four replicate wells per dilution. Heat-inactivated serum pools were two replicate wells per pool, with 50 μl per well. Antisera were loaded into the plate first and diluted. Wells that did not contain antisera, i.e., virus dilutions and cell controls, received 50 μl of diluent. The working dilution of virus containing 100 $TCID_{50}$ in 50 μl was added, and the plates were incubated at 37°C in 5% CO_2 for 1 h. During the incubation period A549 cells were trypsinized and resuspended at 5×10^4 cells per ml. Cell suspension (100 μl per well) was added to the plates, the solutions were mixed, and the plates were incubated at 37°C in 5% CO_2 for 7 days. Each plate contained the following controls: four wells of virus control solution containing the working dilution of virus, diluent, and cells; four wells of cell control solution containing only cells and 100 μl of diluent; and four blank control wells for the spectrophotometer, containing 200 μl of diluent. We have found that neutral red solutions will bind to dry plastic wells, producing a high background in blank wells, which is needed to calibrate the spectrophotometer, unless they are filled with diluent during incubation and prior to staining. Each assay contained a back titration of the virus used.

After 7 days, 100 μl of medium was removed from the wells and discarded. A 100-μl portion of a 1:5,000 solution of Finter's neutral red, a vital stain (13), in isotonic phosphate-buffered saline (PBS) was added, and the plates were incubated for 1 h at 37°C in 5% CO_2. All medium was removed, and the wells were gently washed twice with 150 μl of PBS. A final solution of acid alcohol (50% ethanol, 1% acetic acid in distilled water) was added (100 μl per well). The A_{550} of solubilized dye was read on an E12e Bio-Kinetics Reader (Bio-Tek Instruments, Winooski, Vt.) interfaced with an IBM-type personal computer equipped with Mirror II cross-talk communications software (SoftKlone Distributing Corp.). Data were imported into Lotus 1-2-3 spreadsheet templates customized to plot neutralization as a percentage of cell control absorbance, to determine endpoint virus and serum titers, and to identify intersecting pool combinations.

In a microtiter assay cell density is a critical parameter, governing both virus titer and assay kinetics (4). Within a 10-fold range, from 2×10^3 to 2×10^4 cells per well, the smaller the number of cells, the greater was the amount of virus expression. Stock virus $TCID_{50}$ differed by 2 log units between the highest and lowest densities. However, a minimum number of cells are necessary to provide sufficient dye uptake to produce a workable range of values. A wider range of possible values reduces the error inherent in any single datum point. We selected 5×10^3 cells per well as a compromise. Attempts to use preformed cell monolayers resulted in unacceptably low virus titers. Virus endpoint titers were calculated as the reciprocals of the highest dilutions producing absorbance values less than or equal to 50% of cell control absorbance values, i.e., comparable to that of the $TCID_{50}$ commonly used.

In order to determine how closely an optical density value compared with the degree of CPE, virus titrations of three prototype AV serotypes in microtiter format were observed

TABLE 1. Comparison of AV prototype virus titers and homotypic and heterotypic neutralization titers in the CMN and in standard tube methods

Virus type[a]	$TCID_{50}$[b]		Reciprocal of antiserum neutralization titer[c]							Homotypic tube neutralization titer
	CMN	TN[d]	AV 2	AV 7	AV 35	AV 44	AV 47	AV 48	AV 49	
AV 2 (AD6)	5.5	6.0	2,048	–	–	–	–	–	–	256
AV 7 (Gomen)	4.0	4.5	–	3,200	–	–	–	–	–	1,024
AV 35 (Holden)	4.0	4.5	–	–	100	–	–	–	–	128
AV 44 (1584)	4.0	3.5	–	–	–	1,600	–	–	–	2,048
AV 47 (1601)	3.5	4.5	–	–	–	–	1,600	–	–	1,024
AV 48 (T85-884)	3.5	4.5	–	–	–	(160)	–	12,800	–	4,096
AV 49 (T87-677)	3.5	4.5	–	–	–	–	(20)	–	6,400	2,048

[a] AV serotype prototype strains.
[b] $TCID_{50}$ values are reciprocals of the log dilutions.
[c] Titers are reciprocals of the highest dilutions producing 50% or greater neutralization. –, titer of <10. Corresponding cross-reactions are given in parentheses.
[d] TN, tube method. Virus and antiserum titers were determined at 14 days of culture in human fetal diploid kidney cells (14).

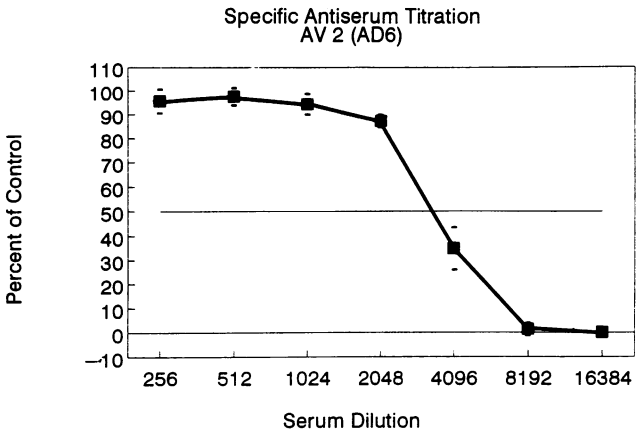

FIG. 2. Neutralization curve plotted from absorbance values from the spectrophotometer and plotted in Lotus 1-2-3 equipped with WYSIWYG. The x axis indicates the reciprocal of the serum dilution. The 50% neutralization endpoint titer was 2,048. Error bars indicate 1 standard deviation.

TABLE 2. Clinical isolates identified by CMN

VRDL No.[a]	Diagnosis[b]	Source	Serotype(s)[c]
17-009	AIDS	Stool	20 and 32
17-010	Kidney Tx	Urine	27 (×37)
17-011,012	AIDS	Colon biopsies	46
17-013,014	AIDS	Rectal biopsy, swab	23
17-015	Conjunctivitis	Eye swab	3
17-106	AIDS	Rectal swab	2
40-001,002,003	AIDS, CMV	Bronchoalveolar lavage, lungs	9 (×8)
40-004	AIDS	Bronchoalveolar lavage	22
40-005	AIDS	Biopsy	26
40-006	AIDS	Rectal swab	Not AV 1–49
40-007,008	Bone marrow Tx	Urines	34 and 35
40-009	AIDS, CMV	Urine	9

[a] VRDL, Viral and Rickettsial Disease Laboratory, California Department of Health Services.

[b] Tx, transplant; CMV, cytomegalovirus.

[c] By convention, serotypes with cross-reaction titers within eightfold of the prototype homotypic titer follow the primary serotype. Serotypes of isolates with lower-level cross-reactions are enclosed in parentheses.

and scored for CPE before being stained for colorimetric analysis. CPE scoring was done according to conventional methods (14). Figure 1 shows the results of plotting CPE versus absorbance, expressed as a percentage of cell control absorbance. By linear regression (Lotus 1-2-3) correlation was exceedingly high ($r = \mid 0.977625 \mid$), demonstrating that there is a direct relationship between the amount of dye absorbed and the amount of CPE. This lends the colorimetric assay a distinct advantage over CPE determination when heteroploid cell lines which tend to round and pile up over time, making the extent of CPE difficult to judge, are employed.

Table 1 shows the comparison of the CMN with standard tube methods to determine the $TCID_{50}$ of stock virus preparations and to determine homotypic and heterotypic neutralization titers. The tube method used rolling-tube human fetal diploid kidney cell cultures observed for 14 days (14). Virus titers determined by the two methods were comparable. Serum titers in the microtiter assay tended to be as high as or higher than those in tube assays, as has been noted elsewhere (3). Figure 2 is a neutralization curve plotted in Lotus 1-2-3 from the data obtained from the homotypic neutralization of AV 2 in Table 1. Serum titers were calculated on the basis of a 50% neutralization endpoint, but as with a plaque assay, the endpoint may be set at 75%, 80%, or such levels as the application demands.

Serotyping clinical isolates is a two-step process. Each isolate is tested in the intersecting pools and then in a serum titration of the prototype antisera indicated by the pool scheme. The results produced by using the CMN to serotype clinical isolates are shown in Table 2. These AV isolates represent the submissions to this laboratory by a single laboratory in a hospital with a large population of AIDS patients within an 18-month period. As has been noted elsewhere (6, 9, 18), a wide variety of serotypes as well as intermediates were seen in the AIDS patients.

The serotyping of AV requires, first and foremost, standardized, reliable reagents (8). Antisera with known reactivities are essential for reproducible results which can then be compared with those of other laboratories. If such reagents are employed, it is only a matter of recalibration when they are applied to a different assay system. As has been noted, antiserum titers can be higher in microtiter assays, sometimes more than 10-fold higher (3), making microtiter assay systems extremely conservative of reagents. By automating the reading and quantitation of viral effects, labor and time are also saved, and increased objectivity is obtained. This approach has already been applied to the determination of human immunodeficiency virus neutralizing antibody titers (13) and to the screening of large population groups for neutralizing antibody to newly described AV serotypes (1), but it clearly has a wider range of applications. As applied to the determination of serum neutralization, this assay approaches the quantitation and sensitivity of a plaque reduction assay. In specific cases, such as seroepidemiological studies, antibody levels below the 50 to 80% cutoff used to determine a serum titer may be significant as evidence of past or low-level infection. It would be possible to use this assay for large-scale screening of sera or monoclonal antibodies, obtaining a numerical value that would reflect the amount of neutralizing antibody present in each serum specimen tested.

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