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Microneutralization Test for Respiratory Syncytial Virus Based on an Enzyme Immunoassay

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Virus infectivity and antibody neutralization titers for respiratory syncytial virus were determined in cell cultures in microtiter plates. After an appropriate incubation period, the cells were fixed, and an enzyme-linked immunosorbent assay was performed directly in the microtiter plates for detection of virus. Results could be read and recorded automatically, which is especially helpful when running large numbers of tests.

The antigenic characterization of respiratory syncytial virus (RSV) strains requires testing with a variety of polyclonal sera and monoclonal antibodies produced against representative strains of RSV. Virus neutralization tests are an integral part of these studies, and large numbers of assays often must be performed simultaneously. We developed a microneutralization test in which virus replication is detected by enzyme-linked immunosorbent assay (ELISA). With this technique, we are able to run large numbers of neutralization tests and to automate the reading and recording of results.

The Long strain of RSV (2) was grown in HEp-2 cells under Eagle minimal essential medium (MEM) with 2% fetal bovine serum (FBS). The virus was harvested at a cytopathic effect (CPE) of 3 to 4+, sonicated for 10 s at 50% power with a Microson ultrasonic cell disruptor (Heat Systems—Ultrasonics, Inc., Farmingdale, N.Y.), divided into portions, and stored at −70°C. The same preparation of virus was used for a series of tests, but a fresh sample was used for each test run.

The neutralization tests were performed in 96-well, flat-bottomed, tissue culture, microtiter plates (catalog no. 3596; Costar, Cambridge, Mass.). Serum or a monoclonal antibody (MAb) in the form of ascites fluid, which had been heat inactivated at 56°C for 30 min, was added to duplicate wells, and serial fourfold dilutions were performed in the microtiter plates. All dilutions were in MEM–2% FBS, and the final volume was 75 μl per well. Approximately 60 50% tissue culture infective doses of virus in 25 μl of MEM–2% FBS then were added to each well, and the mixture was incubated for 2 h at 4°C.

Approximately 15,000 HEp-2 cells in 100 μl of MEM–5% FBS were added to each well, and the plates were wrapped in cellophane and incubated at 35.5°C in a humidified CO_2 incubator for 3 days. The plates were fixed by aspirating the contents of the wells, washing three times with phosphate-buffered saline (PBS) at pH 7.2 with 0.5% Tween 20, adding 75 μl of an 80% (vol/vol) solution of acetone-PBS, and incubating for 15 min at 4°C. After the incubation period, the contents were aspirated, and the plates were air dried.

The ELISA was performed on the same day as the fixation, or the plates were stored overnight at 4°C and the ELISA was performed on the next day. For the ELISA, the wells were precoated with 200 μl of PBS with 0.5% gelatin for 30 min at 35°C, the contents were aspirated, the wells were washed with PBS (pH 7.2)–0.5% Tween 20, and 75 μl of bovine anti-RSV serum (BaRSV) (Burroughs Wellcome Co., Research Triangle Park, N.C.) diluted in PBS–0.5% gelatin plus 0.5% Tween 20 and 2% normal goat serum was added and incubated for 1 h at 35.5°C. The contents were aspirated, the wells were washed, and 75 μl of peroxidase-conjugated, goat anti-bovine immunoglobulin G (IgG) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) diluted in PBS–0.5% gelatin–0.5% Tween 20–2% normal goat serum was added and incubated for 1 h at 35.0°C. The contents of the wells were aspirated again, the wells were washed, and 125 μl of substrate (0.4 mg of *o*-phenylenediamine dihydrochloride per ml, 0.015% H_2O_2) in 0.15 M citrate phosphate buffer (pH 5.5) was added and incubated at room temperature for 40 to 45 min. The reaction was stopped with 3.5 M HCl, and the A_{490} was read with an MR580 Microelisa Auto Reader (Dynatech Laboratories, Inc., Alexandria, Va.). Each dilution of antibody was run in duplicate, and each plate included control wells with uninfected cells, a back titration, i.e., titration of the virus inoculum in MEM–2% FBS, and a titration of a preimmune, or nonneutralizing, antibody. An absorbance reading of ≥3 standard deviations above the mean of 15 control wells was considered to be evidence of virus replication. The dilutions of BaRSV (1:1,000) and goat anti-bovine IgG (1:5,000) used throughout the study initially were determined by checkerboard titrations.

The fixation procedure was chosen from several which we evaluated for plate clouding and ratio of absorbance in infected cells over absorbance in uninfected cells when MAbs against the nucleoprotein (N), fusion protein, and large glycoprotein were the detector antibodies. A peroxidase-conjugated, goat anti-mouse IgG antibody (Kirkegaard and Perry Laboratories, Inc.) was the conjugate for these tests. Acetone-PBS mixtures with ≥90% acetone clouded the plates; mixtures with ≤85% acetone did not. A 3% (vol/vol) formaldehyde-PBS mixture gave decreased absorbance ratios for some N MAbs. Incubation of an 80% (vol/vol) solution of acetone-PBS for 10, 15, or 30 min at 4°C or for 10 or 15 min at room temperature gave similar absorbance ratios, but when the mixture was incubated for 30 min at room temperature, the absorbance ratios for some N MAbs were again decreased. Thus, we chose to fix the plates with an 80% (vol/vol) solution of acetone-PBS for 15 min at 4°C. With this procedure, neither the fixation process nor the presence of cells in the plates altered the absorbance readings. The mean absorbance of 96 wells with substrate, no cells, and no fixation was 0.010; the mean absorbance of 96 wells with cells and fixation was 0.013. The median

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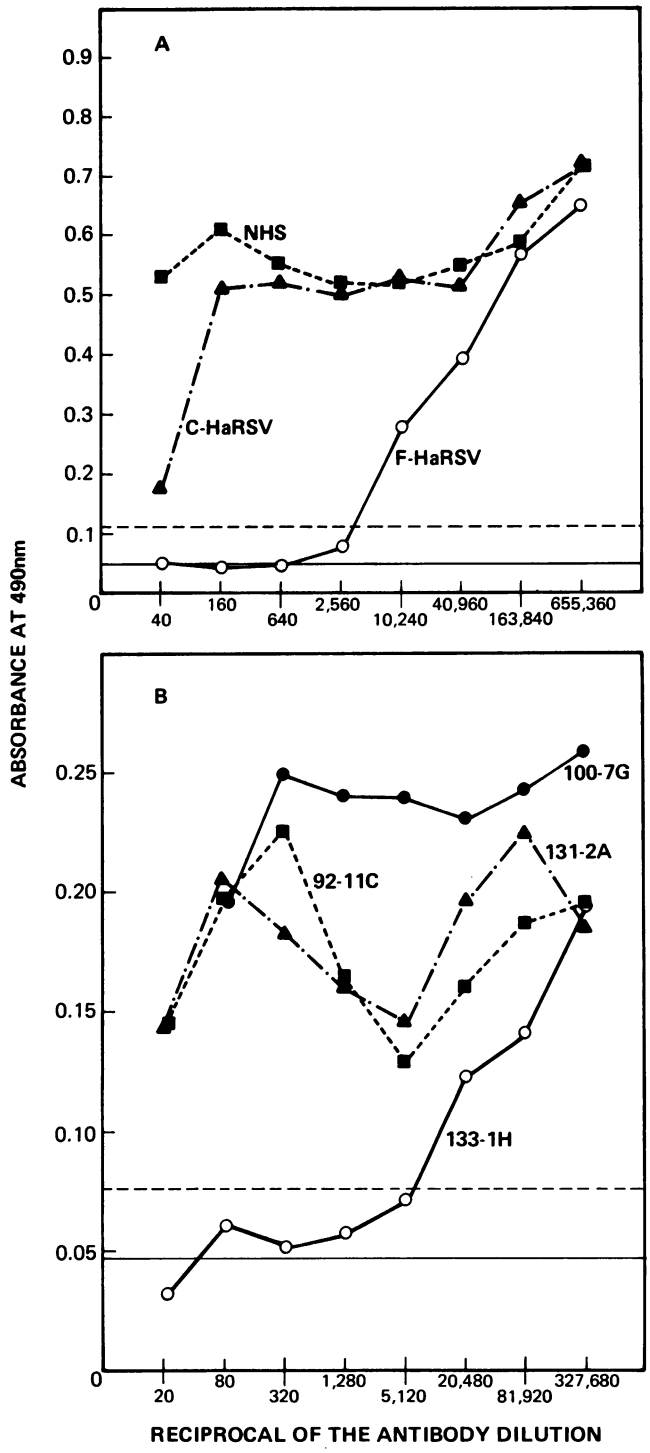

FIG. 1. Neutralizing antibody titration based on an ELISA. The neutralization test was performed in microtiter plates, and virus replication was detected by ELISA. The detector antibody was a BaRSV, the conjugate was a peroxidase-conjugated goat anti-bovine IgG antibody, and the substrate was *o*-phenylenediamine dihydrochloride. An absorbance reading of 3 standard deviations above the mean background was considered evidence of virus replication, and an absorbance reading of 3 standard deviations below the mean of a nonneutralizing antibody was considered to be evidence of reduced virus replication. (A) The three sera tested were a normal horse serum (NHS) and F-HaRSV from Flow Laboratories, Inc., and C-HaRSV from the Centers for Disease Control. The back-titer of virus is 1:64, the mean background is 0.044 (solid line), and 3 standard deviations above the mean is 0.118 (dashed line). (B) The four MAbs are directed against the RSV N (100-7G) and fusion protein (92-11C, 131-2A, and 133-1H). The back-titer of virus is 1:64. The mean background is 0.046 (solid line), and 3 standard deviations above the mean is 0.077 (dashed line). The mean of MAb 100-7G is 0.235, and 3 standard deviations below the mean is 0.170. The 1:20 dilution of MAb 100-7G gave an inadequate test result and is excluded from the figure.

absorbance of uninfected control cells from 10 test runs over 6 months was 0.059, with a range of 0.032 to 0.070.

Four polyclonal anti-RSV sera and two preimmune sera were tested on three separate days. The six sera were a horse anti-RSV serum (F-HaRSV) and normal horse serum from Flow Laboratories; a horse anti-RSV serum from the Centers for Disease Control, Atlanta, Ga. (C-HaRSV); the BaRSV (described above) and a normal bovine serum from Burroughs Wellcome; and a human convalescent-phase serum [LA(12/84)]. Four previously described MAbs against RSV (1) were also tested. One, 100-7G, is against N, and the other three, 92-11C, 131-2A, and 133-1H, are against the fusion protein.

The four polyclonal sera had a range of neutralizing titers. C-HaRSV and BaRSV had low neutralizing titers, LA(12/84) had a moderate titer, and F-HaRSV had a high titer (Table 1 and Fig. 1). For the same serum and virus inoculum, the median difference in neutralizing antibody titer between test runs was 8-fold, with a range of 1- to 128-fold. Much of this difference could be attributed to day-to-day variation in the back titer of virus. The neutralizing antibody titers for a given serum were inversely correlated with the back titer of virus. The coefficients of correlation were −0.79 for F-HaRSV, −0.79 for C-HaRSV, −0.46 for BaRSV, and −0.82 for LA(12/84). Within a test run, the median difference in the back titer for the same virus inoculum was 1.5-fold, and the range was 1.4- to 3.0-fold. Between test runs, the median difference in the back titer for the same virus inoculum was 4.7-fold, and the range was 1.0- to 16-fold.

The titers by tissue culture ELISA were compared with those obtained by reading CPE. Two investigators independently read the microtiter plates for CPE just before the plates were fixed for the ELISA test, and the CPE titer by each investigator was compared with the corresponding ELISA titer. Among 20 comparisons of back-titer determinations by ELISA and CPE analysis, the titers were within 2-fold of each other for 11 (55%), the titer by CPE was ≥4-fold higher for 5 (25%), and the titer by ELISA was ≥4-fold higher for 4 (20%). Among 63 comparisons of antibody titer determinations by ELISA and CPE analysis, the CPE for one test of one serum sample was considered to be unreadable by one of the investigators, the titers were within 2-fold of each other for 30 (48%), the titer by CPE was ≥4-fold higher for 19 (30%), and the titer by ELISA was ≥4-fold higher for 14 (22%). The distribution of higher and lower titers by ELISA was not significantly different from that by CPE.

One of the four MAbs, 133-1H, neutralized RSV, and one, 100-7G, did not, as expected (Fig. 1). The absorbance readings for 100-7G were essentially identical to those for diluent alone (data not shown). Although MAbs 131-2A and 92-11C did not neutralize the virus, they did significantly decrease the absorbance at some of the intermediate dilutions. This corresponded to a decrease in CPE and suggested

TABLE 1. RSV serum neutralization titers by a tissue culture ELISA

Test run (dilution)	Titers[a] on:					
	Plate 1			Plate 2		
	Back-titer of virus	F-HaRSV	C-HaRSV	Back-titer of virus	BaRSV	LA(12/84)
1 (1:400)	1:128	1:2,560	<1:40	1:43	1:320	1:320
1 (1:1,600)	1:16	1:20,480	1:160	1:11	1:40	1:2,560
2 (1:400)	1:85	1:5,120	<1:40	1:43	<1:40	1:640
2 (1:1,600)	1:32	1:10,240	1:80	1:21	1:40	1:1,280
3 (1:400)	1:8	1:40,096	1:320	1:11	1:2,560	1:5,120

[a] The back-titer (titer of virus inoculum) and antibody neutralization titers were calculated by the Karber method. Wells were considered positive when they had an absorbance reading by ELISA 3 standard deviations above the mean of the wells with uninfected cells.

that MAbs 131-1A and 92-11C partially neutralized the virus. This finding is under further study.

This tissue culture ELISA was a convenient and practical microculture test. It gave consistently low background absorbance readings and titers similar to those obtained by reading CPE. Its construction permitted us to perform multiple neutralization tests simultaneously. Others have obtained good results when radioimmunoassays (5, 6) and immunofluorescence assays (7, 9, 10) were used to detect virus replication and to determine viral infectivity and antibody neutralization titers. However, these tests were not performed in microtiter plates. Several investigators have described tests similar to this test, in which virus replication in microtiter plates was detected by immunofluorescence assays (8), immunoperoxidase staining (3, 4), or ELISA (4). In the test with ELISA, the substrate solution was transferred to a second microtiter plate before absorbance was read (4). The major advantage of the ELISA test described here over these other tests and tests in which CPE was used to detect viral replication is that results can be read and recorded automatically from the plate in which the test is performed. This simplifies the test procedure and eliminates one source of recording errors. In the case of CPE analysis, it also eliminates the fatigue and subjectivity associated with reading large numbers of tests.

Another advantage of ELISA over reading CPE is its specificity. The ELISA results are not subject to nonspecific changes in cells which can make accurate readings of CPE difficult. The specificity of the ELISA test is independent of changes in the cells and, instead, is dependent on the quality of the reagents and the correct performance of the test. When good quality reagents are available, the test is technically easy to perform and monitor and should give results specific for viral replication.

This tissue culture ELISA has the potential for application to a wide variety of serologic test systems in virology. For example, we adapted this system to test for RSV-specific IgG antibodies in human sera and to study competitive binding between MAbs. It should also be applicable to test systems in which the virus replicates but gives indistinct or no CPE.

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