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# Microneutralization Test for Detection of Rhinovirus Antibodies[1] (37876)

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The neutralization test remains the standard tool for determining titer of specific antibodies for all rhinovirus types (1). The method requires use of a small dose of virus to achieve needed sensitivity. Tests are generally performed in tube cultures of a cell system such as WI-38 in which cytopathic effect can easily be detected (2). The technique is highly sensitive and reproducible but is expensive in terms of cell cultures required and media and sera used.

A simple micromodification of the procedure would clearly be of importance if it retains the sensitivity of the original technique. The present report describes the development of such a method, which can be performed with standard microtiter equipment. An added advantage is that endpoints can be read macroscopically and the plates preserved for later reference.

*Materials and Methods. Sera and viruses.* A total of 147 sera collected during the study of respiratory infections in Tecumseh, Michigan, were tested for antibody content. In general, three specimens had been collected from each individual during the course of 1 year's surveillance (3). The sera were inactivated at 56° for 0.5 hr and were diluted 1:2 in Hanks' Balanced Salt Solution (BSS). Rhinoviruses used had originally been isolated from individuals in the Tecumseh community in WI-38 cells and had been identified as types 12 and 15 (4).

*Microprocedure.* HeLa (Rhino) cells, originally obtained from Grand Island Biological Co., were propagated serially in our laboratory; for growth, Eagle's Minimum Essential Medium (MEM) in Hanks' salts was used, supplemented with 10% calf serum and with 100 U of penicillin and 100 μg of streptomycin per ml. Bottle cultures were trypsinized after a full cell sheet had formed, usually following 3–4 days of growth. These cells were used to make either microplates or tube cultures. The tube cultures were employed for adaptation of rhinoviruses to the HeLa (Rhino) cell system for later use in the microprocedure (5).

Tests were performed in sterile flat-bottom plastic microplates. Diluent used throughout was Eagle's MEM in Earle's salts; supplements were 7.5% calf serum inactivated at 56° for 0.5 hr and 100 U of penicillin and 100 μg of streptomycin per ml. Diluent was added to the wells in 0.025-ml amounts and the human serum already at 1:2 was further diluted serially using microloops calibrated to contain 0.025 ml. To the residual 0.025 ml of diluted sera in each well, 0.025 ml of virus was added. The viruses had been diluted to contain 32-100 $TCID_{50}$/0.025 ml. The plates were loosely covered; since incubation of the serum–virus mixture was found to increase antibody titer by 1–3 dilutions, they were then placed in a $CO_2$ incubator at 33° for 1 hr. Thereafter, trypsinized HeLa (Rhino) cells were diluted to 100,000–120,000 cells/ml; 0.1 of these cells were added to each well of the plate. Finally, 0.05 ml of diluent was added to each well. Always included in each test were a titration of virus used and serum and cell controls.

Plates were loosely covered and placed back into the $CO_2$ incubator at 33°. After

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6 days of incubation, the media in the plates were decanted, and 1 drop of a solution of crystal violet was added to each well; 1 liter of the solution had been made up of 7.5 g of crystal violet dissolved in 50 ml of 70% ethyl alcohol, 250 ml of formalin, and the remainder of distilled water (6). Cells were stained for 5–10 mins, washed three times with distilled water, and residual water drained from the wells. The test was read after the wells had dried overnight; the stained preserved cell sheet could be best seen at this time. A scoring system from 1 to 4 was used in recording viral activity, based on the portion of the cell sheet that had been destroyed. A well was considered virus positive if the score for that particular well was 3 or more.

*Tube neutralization test.* The tests were performed in tube cultures of WI-38 cells, as previously described (7). Virus propagated in WI-38 cells was diluted to contain 10–32 $TCID_{50}$/0.1 ml. To 0.3 ml of diluted virus, 0.3 ml of the human sera previously diluted 1:2 was added. Following incubation at room temperature for 1.5 hr, 0.2 ml of each serum–virus mixture was inoculated into two cell culture tubes. The tubes were placed on a roller drum and incubated at 33°. End-points were read microscopically when the cytopathic effect in the titration of virus included in the test had reached the expected level; neutralization was considered to have taken place only if no cytopathic effect was observed in a particular serum–virus cell culture.

*Results. Replicate testing of sera by the microtechnique.* The microtechnique was performed in flat-bottomed plates, and endpoints were read macroscopically after the cell sheets in the wells were stained. Therefore, microscopic examination of the test was not necessary. A stained preserved plate containing a virus titration, cell and

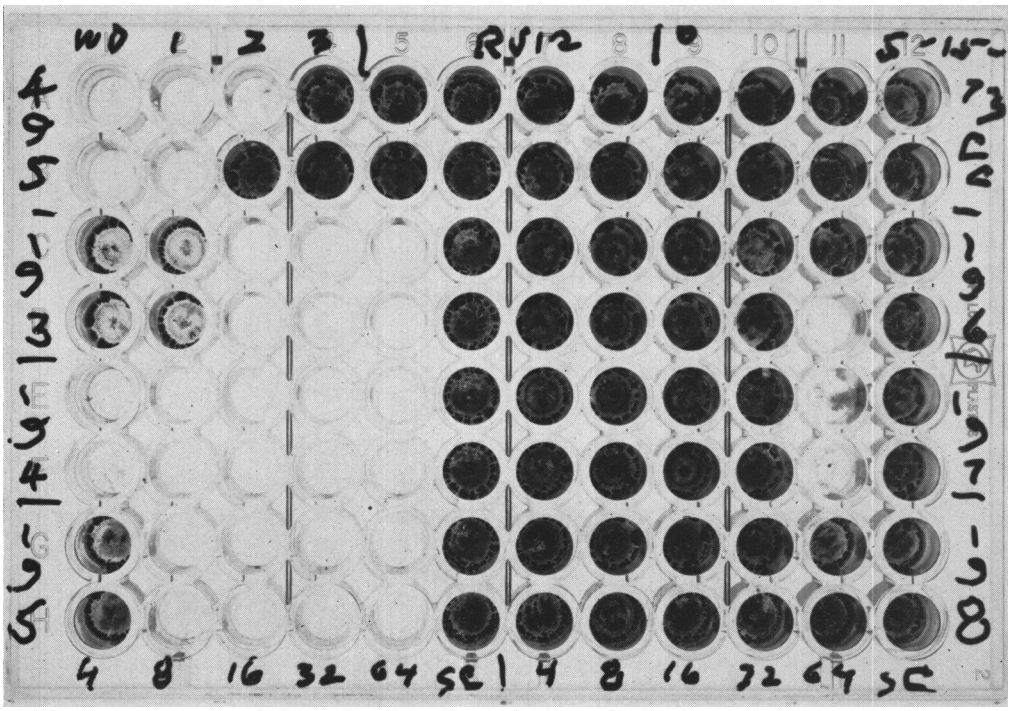

FIG. 1. A microplate in which the cell sheet was stained and preserved. In the upper left, a duplicate titration of pool 495 of rhinovirus type 12 shows that 100 $TCID_{50}$ has been used. The rest of the upper two rows contains cell controls. Six sera were titrated in duplicate; each titration ended in a serum control (SC). Endpoints are: serum No. 193, 1:8; No. 194, <1:4; No. 195, 1:4; No. 196, 1:64–1:32; No. 197, 1:32; No. 198, ≥1:64.

serum controls, and tests on six sera are shown in Fig. 1. The dark-stained wells indicate growth of cells and lack of virus growth. The virus titration shows that 100 $TCID_{50}$ were used in the test.

The reproducibility of the microtechnique was first investigated. Each serum was run in duplicate within a test against rhinovirus type 12. On a separate occasion, it was rerun, again in duplicate, against type 12. The entire procedure was repeated with rhinovirus type 15. Sera were identified by code numbers and results of each test recorded independently.

The initial comparison was within the antibody titration performed on the same serum in the same test. Since the 147 sera had been tested in duplicate on two separate occasions against two viruses, there were 588 separate determinations. In 565 cases (96.1%), results were identical. In 20 cases (3.4%), the difference was one dilution and in three cases (0.5%) two dilutions or more. For purposes of further comparisons, a mean value was used for the few tests in which a discrepancy occurred.

The relation of antibody titers observed when sera were tested twice in totally separate tests against the same virus was next examined. Results are given in Table I. This comparison was an extreme test of reproducibility since the actual virus dose in tests run on different days is not always the same. Thus, it was of note that the differences in titer were less than two dilutions for 93.2% of sera tested against type 12 and 90.5% of sera against type 15. The larger changes in titer were clearly related to the amount of virus that actually was used in the particular test.

TABLE I. Reproducibility of Antibody Titers when 147 Sera were Tested Twice against Rhinovirus Type 12 and against Rhinovirus Type 15.

| Difference in titer between replicate tests | Type 12 | | Type 15 | |
|---|---|---|---|---|
| | No. | % | No. | % |
| None | 116 | 78.9 | 123 | 83.7 |
| One dilution | 21 | 14.3 | 10 | 6.8 |
| Two dilutions | 9 | 6.1 | 6 | 4.1 |
| Three dilutions | 1 | 0.7 | 5 | 3.4 |
| Four dilutions | 0 | 0.0 | 3 | 2.0 |
| Total | 147 | 100.0 | 147 | 100.0 |

TABLE II. Prevalence of Antibody against Rhinovirus Types 12 and 15 in 147 Sera.

| Antibody titer[a] | Type 12 | | Type 15 | |
|---|---|---|---|---|
| | No. | % | No. | % |
| <1:2 | 76 | 51.7 | 107 | 72.8 |
| 1:2 | 10 | 6.8 | 7 | 4.8 |
| >1:2 | 61 | 41.5 | 33 | 22.4 |
| Total | 147 | 100.0 | 147 | 100.0 |

[a] Fifty percent endpoint determined in WI-38 cell culture tubes.

In the above comparison, each serum was considered separately. When the sera obtained from the same individual were examined together, a different situation was observed. None of the differences in results between the two tests had produced a change in interpretation of results for an individual. If there was no four-fold rise in antibody titer by one test, there was no such rise in the replicate test because all sera belonging to the same individual had moved up or down in titer together. If a fourfold or greater rise in antibody titer was found in one test, it was also found in the other test.

*Comparison of microprocedure with standard tube culture method.* With the reproducibility of the microtechnique satisfactorily demonstrated, attention was next directed to its sensitivity. The basis for comparison was a standard method of known sensitivity in detection of serologic conversions in which tube cultures of WI-38 were used.

Results of testing the 147 sera at 1:2 dilution by this method against the two rhinoviruses are given in Table II. The prevalence of antibody against type 12 was approximately twice as high as that against type 15. As is usual in this procedure, a small number of sera were found using the 50% endpoint to contain antibody at 1:2. The series of sera obtained from the same individuals at 6-month intervals was also examined to determine the number of serologic conversions. This has been defined as a rise in antibody titer from <1:2 in one

TABLE III. Comparison of Standard WI-38 Tube Technique and Microplate (HeLa) Technique for Determination of Antibody in 147 Sera against Rhinovirus Types 12 and 15.

| Antibody titer tube technique[a] | Antibody titer microtechnique[a] <1:4 No. | % | ≥1:4 No. | % | Total No. | % |
|---|---|---|---|---|---|---|
| <1:2 | 181 | 98.9 | 2 | 1.1 | 183 | 100.0 |
| 1:2 | 11 | 64.7 | 6 | 35.3 | 17 | 100.0 |
| >1:2 | 14 | 14.9 | 80 | 85.1 | 94 | 100.0 |
| Total | 206 | 70.1 | 88 | 29.9 | 294 | 100.0 |

[a] Results for types 12 and 15 combined.

serum to >1:2 in the next serum. Such conversions were found in one (2%) of the 50 individuals for rhinovirus type 12 but with five (10%) for type 15.

The results of the tube neutralization test were next compared with the results obtained in the microprocedure. Because of similarity in pattern, the data from tests involving types 12 and 15 were combined and are given in Table III. In the micromethod, the lowest serum dilution tested was 1:4; if a serum reproducibly neutralized viral growth at this or higher dilutions, it was placed in the ≥1:4 group. In the tube technique, only a single serum dilution of 1:2 was used. If the titer in tubes was <1:2 for a particular serum, the titer in microplates was nearly always <1:4. However, approximately 15% of sera which had titered at >1:2 in tubes were not found to have antibody at 1:4 in the microprocedure. This may in part indicate that the microtechnique is somewhat less sensitive than the tube technique, but may also relate to the difference in serum dilutions used. The results with the small number of sera titering exactly 1:2 by the tube technique would suggest the latter, since about one-third of them did exhibit antibody at 1:4 in the microplates.

The sera were also compared, in terms of the 50 individuals tested, to determine if differences existed in ability to detect significant changes in antibody titer. For type 12, the single serologic conversion found in the tube technique was not found in the plate test. However, two fourfold rises from pre-existing titer were found in the latter test which could not be detected in the former because of the lack of serial dilutions; two serial falls in titer were also observed. For type 15, 4 of the 5 conversions found in the tube test were confirmed as fourfold rises in titer in the microtechnique. No additional rises from pre-existing titer were found. The difference in sensitivity for conversions may again relate to the inability to use a starting titer lower than 1:4 in the microtest for technical reasons.

*Discussion.* Study of the behavior of rhinoviruses in populations has been hampered by the inability to apply seroepidemiologic methods extensively to the problem. Thus, a reproducible sensitive neutralization test using microdilution equipment would be of great value. The technique herein described has the advantage of employing microloops for dilution of serum, so that many sera can easily be handled. Virus and cells are thereafter added to the wells without the necessity of transferring the serum. In order to allow use of the microloops, the lowest serum dilution that could be tested was 1:4. Some sensitivity was lost by not employing an initial 1:2 dilution, as in the tube neutralization technique, but the ability to test large numbers of specimens in serial dilution appeared more than adequate compensation.

Other microtest methods involving rhinovirus neutralization have either required predilution of sera in tubes and subsequent transfer to special plates, a procedure not applicable to determination of antibody titer (8), or the use of a color endpoint, which can sometimes be indistinct (9). In the present technique, the plates are stained so that extent of cell destruction by virus can be quantitated and endpoints can be easily read macroscopically; as an added advantage, the plates can be stored for future reference and comparison. Rhinovirus isolates and known prototype strains can be quickly adapted to grow to high titer in HeLa (Rhino) cells, which are simpler to maintain and propogate than WI-38 cells (5).

The reproducibility of the technique was quite satisfactory both in tests performed on the same sera on different days and the duplicate dilutions in the same tests. It is therefore conceivable once the procedure is standardized in a particular laboratory that this duplicate dilution in the same test be eliminated. Such a modification would be particularly useful when many sera are being tested against a number of different viruses as part of serologic surveys.

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