A Micro-Method for Assay of Neutralizing Antibodies Against Parainfluenza Virus Types 1 and 3. (31653)

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The advantages of the micro-technique for viral serological studies have been described by Takatsy and Sever(1,2). Adaptations of this technique for detection of neutralizing antibodies have been reported for enteroviruses, adenoviruses, and reoviruses(3-5).

The present communication describes a micro-method for assay of neutralizing antibodies to parainfluenza virus types 1 and 3 in secondary cell culture monolayers grown on disposable plastic plates. In addition, a comparison of the sensitivity, specificity and reproducibility between this technique and the standard roller tube tissue culture method is presented.

Materials and methods. Sera. Sera were collected from adult volunteers or infants age 6 to 12 months who were immunized with formalin-inactivated parainfluenza virus types 1 or 3 vaccines.† All sera were heat-inactivated at 56°C for 30 minutes.

Media. Hanks' balanced salt solution with 0.5% buffered gelatin and antibiotics were used for virus dilutions. The media used in the micro-method consisted of either Eagle's diploid GBI (Grand Island Biologicals) or Leibovitz-15 medium(6) supplemented with 2% heat-inactivated agamma calf serum, 0.2% rabbit SV5 antiserum, 2% IM L-glutamine, penicillin 250 U/ml, streptomycin 250 μmg/ml and amphotericin B 5 μmg/ml. In the roller tube test, cells were maintained on Eagle's GBI diploid media with all the above additives except calf serum.

Virus. The same virus strains were used for both the micro and roller tube neutralization tests. Parainfluenza virus type 1 (Strain 20993) was isolated in rhesus monkey kidney culture and passaged in the same system eight times. Parainfluenza virus type 3 (Strain C243) was recovered in rhesus monkey kidney culture and passaged in the same system 2 times.

Cells. In the roller tube test, 7- to 10-day-old primary rhesus monkey kidney cell cultures were used. In the micro-neutralization test, secondary cell cultures were derived from primary rhesus monkey kidney cell monolayers grown with SV5 antiserum in 32-oz prescription bottles. After 7-10 days of growth the monkey kidney cells were removed from the glass bottles by trypsinization, and then resuspended in maintenance medium. The cell concentration was adjusted to 250,000-500,000 cells per ml ($10^{5.4}$-$10^{5.7}$ cells/ml), before addition to the plates.

Plates. In the microneutralization test, titrations and growth of cells and virus were carried out in disposable vinyl plastic plates.‡ Each 4 × 6 inch plate contained 96 U-shaped cups which were designed to hold 0.20 ml of media. Prior to use, the plates were soaked for one hour in a warm detergent solution.§ They were rinsed 5 times in tap water and soaked in 95% ethanol for 30 minutes. The plates were rinsed again 5 times in tap water and 5 times in demineralized distilled water. After drying in an inverted position, the plates were exposed to a 15-watt ultraviolet light for at least 12 hours.

Dropping pipettes and dilution loops. Pipettes and spiral wire loops‡ calibrated to deliver 0.025 ml were used for titrations and the addition of reagents. Both droppers and loops were cleaned and decontaminated by repeated rinsing and boiling in demineralized distilled water.

Microneutralization test. Serial 2-fold dilutions of sera were carried out in duplicate in

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† Prepared by Pfizer Drug Co., under contract PH-43-63-582 with the Vaccine Development Board, Nat. Inst. of Allergy & Infect. Dis.

‡ Cooke Engineering Co., Alexandria, Va.—Plate #220-24.

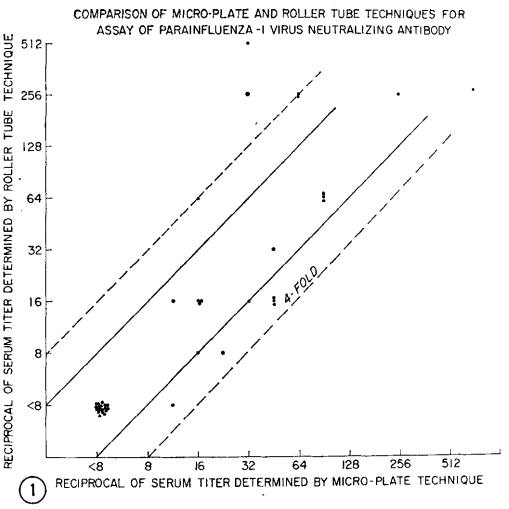
§ Linbro Co. 7×-Solution.

0.025 ml volumes of media with the spiral wire loops. Then, 0.024 ml of the virus dilution calculated to yield 16 to 32 50% tissue culture infectious doses ($TCID_{50}$) was added to each cup. After incubation of the serum-virus mixtures for one hour at room temperature, 0.025 ml of the monkey kidney cell suspension and 0.1 ml of maintenance media was added to each cup. The plates were covered with a loose fitting celluloid sheet and incubated at 37°C in a humidified 5% CO_2 incubator (or in a humidified aerobic atmosphere when L-15 medium was used) for 3 days.

Growth of the virus was detected by testing the cell monolayer covering the hemispherical bottom of each cup for hemadsorption. Plates were inverted and shaken over a large vessel to remove the media. Each cup was filled with 0.85% saline and the plate inverted and drained. A 0.1% suspension of guinea pig red cells in 0.85% saline was added to fill each cup, and the plate was incubated at 4°C for 30 minutes to allow for maximal hemadsorption patterns to develop. The plates were then inverted and shaken to remove non-absorbed red cells and media. They were examined in the inverted position with a light microscope at a 40× magnification.

At this magnification, the microscope field encompassed the entire base of an individual cup, and the hemadsorption pattern was easily readable. The degree of hemadsorption observed was graded 1+ through 4+ according to the percent of the total cell sheet that adsorbed red cells. Because of slight retention or trapping of red cells on irregular areas of the cell sheet, a grade of 1+ or 2+ was not considered as specific hemadsorption. Therefore, 3+ to 4+ hemadsorption patterns were considered as the endpoints for virus infectivity. The test was considered satisfactory if the monkey kidney cell controls showed less than 3+ hemadsorption and the virus controls showed 3 to 4 + hemadsorption patterns. In addition a standard reference antiserum was included in each test.

Roller tube test. Details concerning the standard roller tube tissue culture neutralization test have been described(7). In the roller tube tests, the serum endpoint was considered



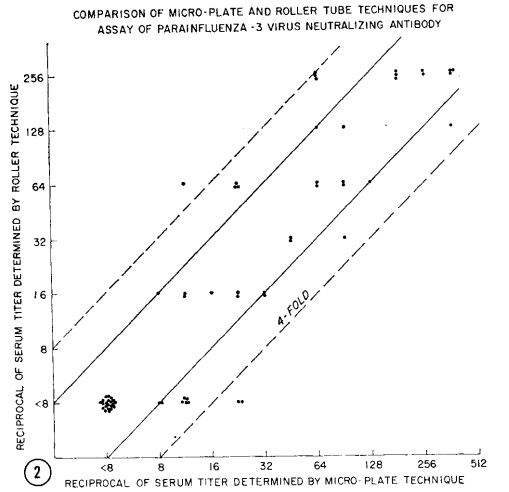


Fig. 1 and 2.

to be the highest dilution at which complete inhibition of hemadsorption was observed. Titers were calculated according to the method of Reed and Muench(8).

Results. A comparison between the micro-plate and roller tube techniques for assay of parainfluenza type 1 and type 3 neutralizing antibody is shown in Fig. 1 and 2. Sera obtained from children aged 6 to 12 months were assayed by both methods. Four-fold or greater differences in titers obtained by both methods occurred in only 5 of the 40 sera tested for parainfluenza-1 type antibody, and only 6 of the 64 sera tested for parainfluenza type 3 antibody differed by this degree. The micro-plate assay appeared to be more sensi-

TABLE I. Variation in Titer of Parainfluenza-1 Virus Neutralizing Antibody in a Human Serum Assayed in 41 Separate Tests.

Geometric mean titer of serum (log_2)	No. of tests with indicated titer
3	
3.5	5
4	17
4.5	8
5	7
5.5	3
6	1
Total	41

tive than the roller tube test in detecting low titers of antibody to parainfluenza type 3 virus.

The variability of titers obtained in a micro-plate assay of parainfluenza type 1 antibody is illustrated in Table I. Duplicate titrations of 161 sera in an individual test gave endpoints that varied by 4-fold or more in less than 5% of the specimens tested. The reproducibility of titers of the same serum obtained by micro-plate tests performed at different times is illustrated in Table II. The same reference serum was assayed 41 separate times, and the spread of observed titers indicated that titers of the same serum tested on two different occasions would differ by 4-fold or more in only 5.8% of the tests (permutations test).

Discussion. The first successful growth of monkey kidney cells on the surface of microplates and its adaptation to the assay of poliovirus antibody were described by Rosenbaum *et al*(3). We found that growth of RMK cells on these plates was improved when the plates were washed in 95% ethanol prior to use. An occasional shipment of plates from the manufacturer failed to support growth of these cells despite attempts to alter the surface with concentrated sulfuric acid, sodium hydroxide or by passing electric current through it. Fortunately all plates within a given shipment have generally been consistent in their ability to support growth of cells.

TABLE II. Variations Between Duplicate Micro-Test Assays of Sera for Parainfluenza-1 Virus Neutralizing Antibody.

Difference in titer between 2 simultaneous titrations (2-fold dilutions)	No.	% of total
0	71	44
2-fold	84	52.2
4-fold	5	3.1
8-fold	1	0.7
Total	161	100

In our laboratory the micro-test has made possible a significant saving of time and expense over the conventional method of assaying neutralizing antibody. A micro-test equivalent to a 3500 roller tube neutralization test was set up by two workers over an 8-hour period, and the test was read 3 days later over a similar time. Because of the ease with which large numbers of sera may be handled at one time, it is likely that data obtained from simultaneous testing of large numbers of sera by the micro-method would be more meaningful than pooled data from several separate tube tests. In addition, the small volume of sera required for the micro-test has made it particularly useful in assaying antibody in serum specimens obtained from children, as well as antibody in various body secretions(9). Recently in our laboratory this test has been used to assay antibody to type 2 parainfluenza virus and mumps virus.

Summary. Assay of neutralizing antibody to types 1 and 3 parainfluenza viruses in microplates was shown to correlate well with titers obtained by the standard roller tube tissue culture method. The micro-test is efficient, sensitive and sufficiently reproducible to be an acceptable method of assay for these antibodies.

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Mechanism of Action of TSH Studied by Organ Culture of Fetal Rat Thyroid Gland.* (31654)

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Using the organ culture technique, we reported recently(1) that addition of TSH§ to the culture medium greatly improved ^{131}I utilization by embryonic thyroid explants obtained from 21-day rat fetuses. Total ^{131}I content as well as incorporation of the isotope into protein-bound iodotyrosines and thyroxine were higher in the presence of TSH; the most striking response observed being the increase in incorporation of ^{131}I into thyroxine. We reported earlier(2) a similar effect of TSH on organ culture of thyroid glands removed from younger fetuses.

It was of interest to determine, under similar conditions, whether TSH stimulation of thyroid hormone production is dependent upon synthesis of proteins. It is shown here that addition of TSH to the medium failed to influence the ^{14}C incorporation of [^{14}C] leucine into protein and that puromycin, at a concentration that inhibited protein formation, failed to abolish the TSH-induced stimulation of ^{131}I incorporation into protein-bound iodotyrosines and thyroxine.

In order to explore the mechanism of action of TSH, we have made use of Tapazole‖ which blocks the protein binding of iodine without affecting the ability of thyroid gland to concentrate iodide(3,4) and of sodium perchlorate which is thought to interfere with the active transport of iodide without affecting its organification(4,5).

Materials. Penicillin G and puromycin were purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio. L-leucine, uniformly labeled with ^{14}C, was obtained from the New England Nuclear Corp., Boston, Mass. Amorphous insulin, 20 units/mg and practically devoid of glucagon (less than 0.0003%) was kindly provided by Otto K. Behrens of Lilly Laboratories, Indianapolis, Ind. The culture medium used in this study was medium TC-199(6), obtained from Hyland Laboratories, Los Angeles, Calif., to which penicillin G was added to provide a concentration of 50 units/ml. The basal culture medium was enriched also by insulin since our previous findings indicated that addition of insulin to the organ culture medium greatly improved the ^{131}I utilization by embryonic thyroid tissue(1,7,8) and also enhanced the incorporation of ^{14}C-labeled L-amino acids into protein(9). Amorphous insulin was dissolved in a minimum volume of 0.003 N HCl. A stock solution of this hormone made to contain 1 mg of insulin per ml of medium was sterilized by passing through a Millipore® filter with an average porosity of 0.45 μ. An amount of the filtrate was added to the medium to yield a concentration of 5 μg of hormone per ml. TSH (Thytropar, obtained from Armour and Co., Kankakee, Ill.) was dissolved in a measured volume of the medium (5 units/ml) and the solution thus obtained was sterilized by passage through the Millipore filter. 2An aliquot of the filtrate was then added to the culture

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§ Abbreviations: TSH for thyrotropic hormone; MIT for monoiodotyrosine; DIT for diiodotyrosine; T_4 for thyroxine.

‖ 1-Methyl-2-mercaptoimidazole.