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COMPARISON OF MEASLES ANTIBODY TITERS MEASURED BY THE MICRO- AND MACRO-METHODS

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SUMMARY Measles neutralizing and hemagglutination inhibition (HI) antibody titers measured using tubes and micro-plates were compared. Neutralizing antibody titration using tubes was the most sensitive, and HI antibody titration using micro-plates gave the lowest titers. The neutralizing antibody titers measured using micro-plates coincided well with the HI antibody titers measured using tubes, and the titers were between those obtained by the two methods cited above. Use of micro-plates for neutralizing antibody titration facilitates serological follow-ups such as those after measles vaccination, because it reduces the size of serum specimens, gives reliable titers of antibodies and allows measurement of low antibody titers which cannot be measured by HI antibody titration.

INTRODUCTION

Takatsy et al. (1954) developed a micro-titration technic, and it is now widely applied to routine serological studies on viral diseases (Sever, 1962; Rosenbaum et al., 1963; Sullivan and Rosenbaum, 1967; Fuccillo et al., 1969).

Hemagglutination inhibition (HI) titration of measles antibody (Rosen, 1961) is a simpler and easier method than neutralizing antibody titration, but low titers cannot be measured by HI titration. In serological follow-ups on children after measles vaccination, an easy, reliable and reproducible titration method for low antibody titers was required. This paper describes such a method and results of comparative studies using tubes and micro-plates.

MATERIALS AND METHODS

1. *Micro-tissue culture equipment*

Plastic, disposable, rigid, micro-plates with 96 " U "-bottom circular wells of Linbro IS-MRC-96 (Linbro Co., New Haven, Conn., U.S.A.) were used for micro-neutralization tests. The plates were washed as described by Sullivan and Rosenbaum (1967) by soaking them in 70% ethyl alcohol for several hours, rinsing them 3 times each in tap water and de-ionized water, draining them thoroughly and drying them in air. The washed and dried plates were sterilized by UV-irradiation for 20 minutes, and covered with heat sterilized aluminium foil.

Micro-pipette droppers (Microtiter, Cooke Engineering Co., Alexandria, Va., U.S.A.) calibrated to deliver 0.025 ml were used to dispense diluent, culture media, cells and sera into wells. Droppers were autoclaved before use.

Diluters calibrated to adsorb and deliver 0.025 ml (Tominaga Works Ltd., Tokyo, Japan) were used for dilution of sera. Made of stainless steel, they can be flamed to sterilize.

2. *Micro-HI equipment*

Plastic, disposable, rigid, micro-plates with 96 " V "-bottom circular wells of Linbro IS-MVC-96 (Linbro Co.) were used for micro-HI tests.

The micro-pipette droppers and diluters used were the same as in micro-neutralization tests.

3. *Cells*

FL cells were used for neutralization tests and challenge virus titration.

4. *Virus*

The Toyoshima strain of measles virus (Toyoshima et al., 1959) cultured in FL cells was used for neutralization tests.

5. *HI antigen*

The antigen used in HI tests was prepared from the Toyoshima strain of measles virus cultured in KB cells by treatment with Tween-80 and ether as described by Norrby (1962). Lyophylized antigen was supplied in ampoules from the Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kagawa.

6. *Tissue culture media*

The growth medium was Eagle's minimal essential medium with 2-fold the standard amounts of amino acids and vitamins (×2 EMEM) containing 10% inactivated calf serum (CS). The maintenance medium was ×2 EMEM containing 2% CS. Antibiotics were added to all media (100 units of penicillin and 100 μg of streptomycin per ml). The pH was adjusted to 7.0–7.2 with 7.5% sodium bicarbonate solution.

7. *Diluent for neutralization tests*

Sera were diluted with 1/100 M phosphate buffered saline without Ca^{++} and Mg^{++} (PBS (−), pH 7.0) containing 0.5% gelatin. Kanamycin was added to a final concentration of 200 μg/ml.

8. *Treatment of sera*

Sera were obtained from 3 patients with measles at various stages of the disease and stored in a deep-freezer at −20 C. Before the test, sera were divided into 2 parts and one part was inactivated by heating at 56 C for 30 min for neutralization tests.

The other part was used for HI tests without inactivation. Volumes of 0.2 ml of sera were added to 0.6 ml of PBS (−) (pH 7.0), and the resulting 4-fold diluted sera were mixed with 0.8 ml of 25% acid-washed kaolin suspended in PBS (−). After vigorous shaking, the mixtures were stood at room temperature (about 20 C) for 20 min with occasional shaking. Then, they were centrifuged for 20 min at 3000 rev/min at room temperature. The clarified supernatants were poured into new tubes and mixed with 0.1 ml of a 10% suspension of African green monkey red blood cells (AGM-RBC) in PBS (−). After shaking, the mixtures of sera and AGM-RBC were stood in an ice-water bath for 1 hr. Then, they were centrifuged for 5 min at 3000 rev/min at room temperature. The supernatants were used for HI tests, and had a final dilution of 8 fold the original sera.

9. *Neutralization tests*

1) Micro-method

Two-fold serial dilutions of 0.025 ml (1 drop) of serum were made by diluter transfer in wells containing 0.025 ml (1 drop) of diluent, and 1 drop (100–300 $TCID_{50}$/0.1 ml) of the challenge virus suspended in the diluent was added to each well with the dropper. Two lines of wells were used for each serum sample. The micro-plate containing the serum-virus mixture was covered with aluminium foil, and incubated at 37 C in an incubator under 5% CO_2–95% air (CO_2 incubator) for 1 hr. Then, 3 drops (0.075 ml) of FL cells (15×10^4 cells/ml) in growth medium were added to each well containing serum-virus mixture. The plate was covered with aluminium foil and reincubated at 37 C in the CO_2 incubator for 2 days to allow the cells to form a sheet. Two days later, the media in the wells were removed with an aspirator (Yamato Kagaku Kikai Co., Tokyo, Japan), and 5 drops (0.125 ml) of maintenance medium were added with the dropper. The plates were sealed with non-toxic plastic tape (" Scotch " brand, Minnesota Mining Manufacturing Co., St. Paul, Minn., U.S.A.) and incubated at 37 C in a conventional incubator. When titration of the challenge virus infectivities gave complete cytopathic effects (CPE) at dilutions of $1 : 10^0$ and $1 : 10^1$ usually 3 to 4 days later, the plate was examined for CPE with an inverted microscope. Neutralizing antibody titers were calculated by the Reed-Muench method (1938)

as the 50 per cent end-point which inhibited the appearance of CPE.

Infectivities of the challenge virus were also assayed in wells. The virus suspension used for the neutralization test was serially diluted 10-fold in conventional tubes with the diluent, and 1 drop (0.025 ml) of each dilution was put in a well containing 1 drop (0.025 ml) of diluent. Five wells were used for each dilution. Subsequent procedures were as for the neutralization test. After 2 weeks observations, the infectivity titer ($TCID_{50}$) was calculated by the Reed-Muench method. The media were renewed if necessary.

2) Macro (tube) method

One ml·(15×10^4 cells) of FL cells in growth medium was seeded into 13 mm × 90 mm tubes. Tubes were fitted with rubber stoppers and incubated at 37 C in a conventional incubator without disturbance for 2 days. Then the medium was replaced by maintenance medium. Volumes of 0.4 ml of 2-fold serially diluted serum samples were mixed with an equal volume of the challenge virus suspension (100–300 $TCID_{50}$/0.1 ml). After incubation at 37 C for 1 hr, 0.2 ml volumes of serum-virus mixture at each dilution were inoculated into 2 FL tubes containing fresh medium. The tubes were closed with rubber stoppers, and incubated at 37 C in a stationary position for 5 to 6 days till complete CPE developed at $1 : 10^0$ and $1 : 10^1$ dilutions of the challenge virus. Neutralizing antibody titers were calculated as in the micromethod.

Infectivities of the challenge virus were assayed using the same tubes as for the neutralization test. A sample of 0.8 ml of the challenge virus suspension used for the neutralization test was mixed with an equal volume of the diluent and incubated at 37 C for 1 hr. After incubation, the 2-fold diluted virus suspension was serially diluted 10-fold in conventional tubes and 0.2 ml volumes of each dilution were inoculated into 5 FL tubes containing fresh medium. After 2 weeks incubation at 37 C the infectivities were estimated by the method of Reed-Muench. The media were changed occasionally.

10. *Hemagglutination inhibition (HI) test*

1) Micro-method

One drop (0.025 ml) of serum, which had been treated with kaolin and AGM–RBC, was serially diluted 2-fold by diluter transfer in wells containing 1 drop of PBS (−). One drop of antigen containing 4 hemagglutination units (HAU) was added with the dropper to each well. After incubation at 37 C for 1 hr, 1 drop of a 0.5% suspension of AGM–RBC was added to the serum-virus mixture and the microplate was again incubated at 37 C for 2 hr. Antibody titers are expressed as the reciprocals of the highest dilution of serum which inhibited hemagglutination completely.

2) Macro (tube) method

A volume of 0.25 ml of the treated serum was serially diluted 2-fold in 10 mm × 70 mm tubes containing 0.25 ml of PBS (−). Then 0.25 ml of antigen containing 4 HAU was added to each tube. After incubation at 37 C for 1 hr, 0.25 ml of 0.5% suspension of AGM–RBC was added to each mixture. Other procedures were as for the micromethod.

RESULTS

Antibody titers measured by the 4 methods are summarized in Table 1. The highest titers were obtained by the neutralization test using tubes (macro-NT). Titers by the HI test using micro-plates (micro-HI) were the lowest. The titers measured by the neutralization test using micro-plates (micro-NT) and those measured by the HI test using tubes (macro-HI) were similar, being intermediate between those measured by the macro-NT and the micro-HI.

The antibody titers measured by the 4 methods are also compared in Fig. 1–4. Good coincidence was obtained between the titers measured by the two neutralization tests, though high titers by the micro-NT tended to be lower than those by the macro-NT (Fig. 1). The titers measured by the micro-NT and the macro-HI also coincided well (Fig. 2). The poorest correlations are shown in Fig. 3 and 4, in which the titers measured by the micro-HI were labile compared with those measured by the macro-, and micro-NT.

TABLE 1. *Comparison of measles neutralizing and hemagglutination-inhibition antibody titers measured by the macro-, and micro-methods*

Serum sample	Neutralizing antibody titer (log_2)		HI antibody titer (log_2)	
	macro	micro	macro	micro
2-1	5.5	4.5	4	3
3-1	5.5	4.5	3	3
1-2	6.5	5.5	6	4
2-2	7.0	6.5	6	4
3-2	7.0	6.5	5	3
5-2	7.0	6.5	5	3
8-2	7.0	6.5	7	6
1-3	8.0	6.5	7	6
2-3	9.0	8.0	8	6
3-3	9.0	7.5	7	5
5-3	9.5	6.5	7	6
8-3	10.5	7.5	9	7
1-4	10.0	8.0	9	7
2-4	10.5	9.0	9	7
3-4	11.5	10.0	8	5
5-4	11.5	9.0	9	7
8-4	12.5	10.0	10	7
Geometric mean	8.7	7.2	7.0	5.2

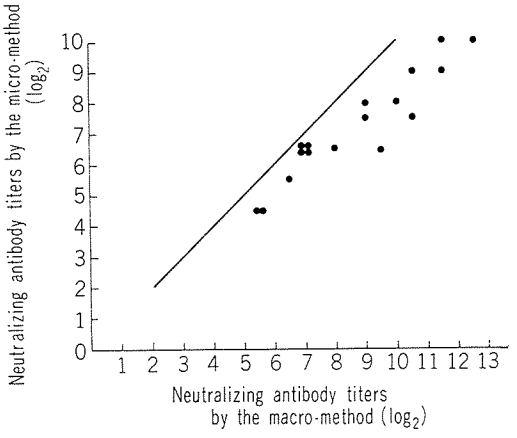

FIGURE 1. *Comparison of measles neutralizing antibody titers measured by the macro- and micro-NT.*

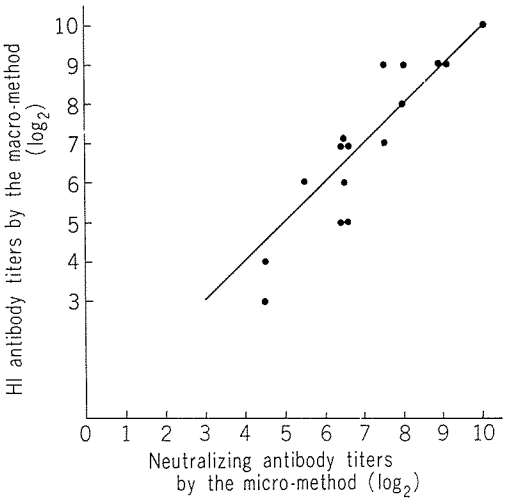

FIGURE 2. *Comparison of measles neutralizing antibody titers measured by the micro-NT and HI antibody titers measured by the macro-HI.*

DISCUSSION

Toyoshima et al. (1965) reported that the overnight macro-NT was 2 to 4 fold more sensitive than the ordinary macro-NT. Their method was suitable for detecting small amounts of antibody such as that in serum after injection of killed measles virus vaccine (Ueda et al., 1966; Tuchinda et all, 1967). However, the macro-NT needed large quantity of serum specimens and was in general laborious.

The HI test developed by Rosen (1961) and the improvement by Norrby (1962) in the method for preparing HI antigen simplified measles antibody titration, but under the conditions reported in this paper, low titers of under 1 in 8 could not be measured because of treatments with kaolin and AGM–RBC.

The micro-techniques developed by Takatsy et al. (1954) have been widely applied in routine serological studies on viral diseases since their application to the metabolic inhibition test by Sever (1962). As reported in this paper, the micro-NT gave reliable results even when compared with the macro-NT. This micro-NT

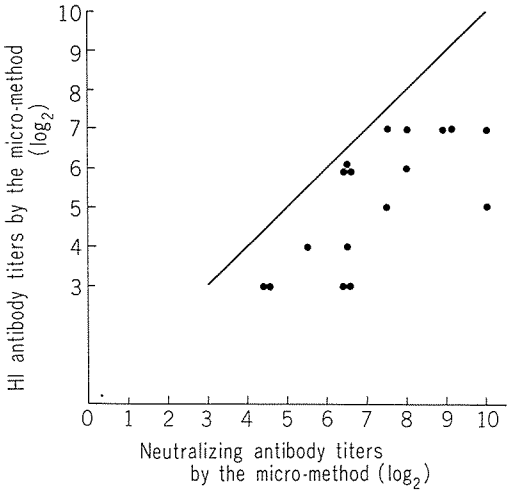

FIGURE 3. *Comparison of measles neutralizing and HI antibody titers measured by the micro method.*

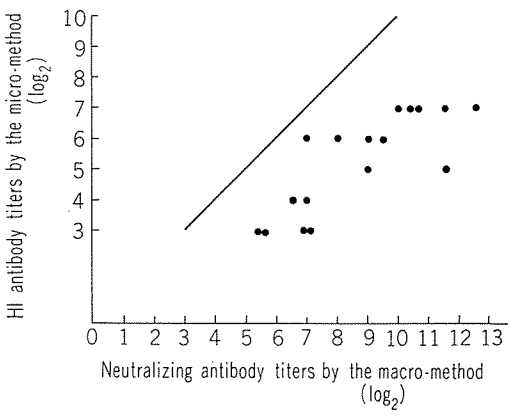

FIGURE 4. *Comparison of measles neutralizing antibody titers measured by the macro-NT and HI antibody titers measured by the micro-HI.*

has the following advantages over other methods: (1) small amounts of reagents including serum specimens are required, (2) rapid dilution of serum specimens can be done in wells, (3) low levels of antibodies can be detected, (4) CPE can be examined quickly and (5) this method is labor saving. So, the micro-NT can be applied in follow-up studies after administration of measles virus vaccine or in serological and epidemiological studies on measles in which antibodies at low titers are important, as reported previously (Ueda et al., 1969a, b).

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