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Comparison of the Hemagglutination Inhibition Test for Measles with Serological Tests and Its Application to a Field Trial*

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SUMMARY

A comparison was carried out between the complement fixation, hemagglutination inhibition and neutralization tests for measles in sera of subjects in various conditions. The hemagglutination inhibition and neutralization tests were more sensitive than the complement fixation test and the antibody titers of the two former were closely related. A booster effect could be observed with the hemagglutination inhibition and neutralization tests in 9 children who had been vaccinated with live measles vaccine in a field trial. These serological responses were hardly detectable by the complement fixation test.

The hemagglutination inhibition test was found to be useful for serological examination in large scale field trials.

INTRODUCTION

In 1954, Enders and Peebles isolated and cultivated measles virus in tissue culture and at the same time they employed the complement fixation (CF) and neutralization (NT) tests for identification of the measles virus. Other investigators applied the CF test for serological surveys of measles antibodies in epidemiological studies (Enders *et al.*, 1957; Ruckle and Rogers, 1957; Black, 1959; Beck, 1959).

Recently, Periés and Chany (1960) described the hemagglutination (HA) and hemagglutination inhibition (HI) tests for measles virus using simian red cells.

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ABBREVIATIONS: CF: Complement Fixation, NT: Neutralization, HI: Hemagglutination Inhibition, HAnin: Hemagglutinin, PBS: Phosphate Buffered Saline (M/15, pH 7.2), TCD_{50}: 50% of Tissue Culture Infective Dosis

The HI test was applied for the examination of measles antibody and was compared with two other serological methods by several investigators (Black and Rosen, 1962; Cutchins, 1962). Though the CF test for measles antibodies was found to be less sensitive than the NT and HI tests (Cutchins, 1962), it was still valuable for screening for antibodies against measles.

Recently it became easy to obtain high potency antigen for the HI test by selection of tissue culture cells for hemagglutinin production and by ether-Tween 80 treatment (Norrby, 1962; Funahashi and Kitawaki, 1963). With this high potency antigen, the HI test could readily be applied in a serological survey of measles.

This paper reports a comparison of the HI test with other serological tests with regard to their sensitivity and reliability with sera obtained from persons who had various histories of measles and the application of the HI test in a field trial carried out in Japan in 1962 in accordance with the WHO measles live vaccine project.

MATERIALS AND METHODS

1. *Virus*

The Toyoshima strain of measles virus adapted to FL cells was employed for neutralization and complement fixation tests. The strain adapted to KB cells was used for the hemagglutination inhibition test, since more hemagglutinin could be obtained from infected KB cells than from infected FL cells.

2. *Tissue culture*

FL cells were suspended in growth medium consisting of Earle's balanced salts solution supplemented with 0.5 per cent lactalbumin hydrolysate (LE) and 15 per cent bovine serum and distributed in 1 ml aliquots in test tubes. One or two days later they were used for the neutralization test. For maintenance of the cells, LE supplemented with 3 per cent bovine serum (MM) was employed.

3. *Antigen for the neutralization test*

The MM of infected FL cultures was replaced with fresh MM when apparent cytopathic changes appeared. About 24 hours later, the infected fluid was harvested, clarified by low speed centrifugation and stored at −20°C after distribution in small tubes. The infectivity of one tube from each antigen lot was tested and lots containing more than 10^5 TCD_{50}/ml were employed for the NT test.

4. *Complement fixing antigen*

FL cultures infected with measles virus were harvested when maximal cytopathic changes were observed. The cell-fluid mixtures were sonicated in a 10 Kc sonic oscillator for several minutes. After low speed centrifugation, they were distributed in test tubes in appropriate volumes and stored at −20°C.

5. *Preparation of hemagglutinin*

KB cells were infected with measles virus at an input multiplicity of about 1. Six or 7 days after infection, the cells and fluid were harvested and stored at −20°C. These materials were

sonicated and after low speed centrifugation, HAnins were sedimented by centrifugation at 36,000 g for 1 hour. Pellets were resuspended in 1/10 volume of M/15 PBS (pH 7.2) and clarified by low speed centrifugation. This material was mixed with Tween 80 (final concentration 2 mg/ml) and then an equal volume of anesthetic ether by vigorous shaking in an ice bath for 15 minutes. The aqueous phase was collected after centrifugation and then the residual ether was evaporated. Relatively pure, highly active HAnin could be obtained by these procedures.

6. *Treatment of sera*

For the NT and CF tests, sera were inactivated at their lowest dilution. For the HI test, kaolin or acetone treatment and monkey red cell absorption were employed. An equal volume of 25 per cent kaolin in PBS and 4 fold diluted serum were mixed and shaken vigorously for 20 min. at room temperature. The mixture was spun at 3,000 rpm for 30 min. and the supernatant was mixed with an equal volume of 5 per cent monkey red blood cells (rbc). After 1 hour at 37°C it was centrifuged and the supernatant was used for the HI test. For acetone treatment, 0.2 ml of serum was mixed with 4 ml of acetone and the mixture was centrifuged. The sediment was washed once with 4 ml of acetone and dried in a desiccator which was evacuated by suction with a vacuum pump for 60 min. Dried material was dissolved in 0.8 ml of PBS and placed in a refrigerator over-night. 0.1 ml of packed monkey rbc was added and then the mixture was treated as described above. Unless otherwise specified, kaolin and monkey rbc treatment was employed. The final concentrations of these treated sera were 1:16 in kaolin treatment and 1:4 in acetone treatment.

7. *Neutralization test*

Serially 2 fold dilutions of sera were mixed with equal volumes of virus containing 10^2 TCD_{50}/0.1 ml and incubated at room temperature for 60 min. 0.2 ml of the mixture was inoculated into each of 4 FL tubes containing 1 ml of freshly changed MM, and the mixtures were incubated at 35°C. Titration of antigen was performed simultaneously with 10 fold serial dilutions. Five tubes were inoculated with 0.1 ml at each dilution and observed for 14 days. The NT titers of the sera were calculated by Reed and Muench's method, after careful microscopic observation at the time when all 5 tubes inoculated with 10^{-1} of antigen showed cytopathic changes.

8. *Complement fixation test*

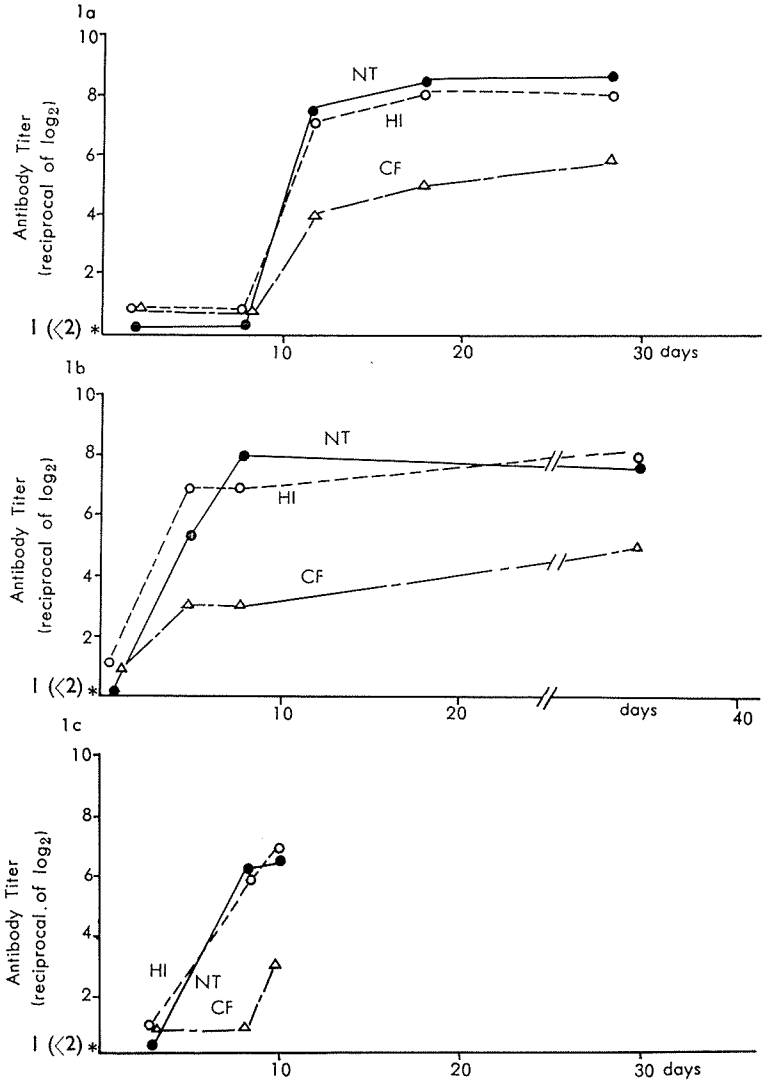
The drop technique using plastic trays was employed. 0.02 ml of 2 fold serially diluted sera were transferred into plastic trays using a syringe for the micro-CF test (Hirasawa). Equal volumes of antigen containing 2 units and 0.04 ml of complement containing 2 full units was added. After incubation in a refrigerator for about 18 hours, 0.04 ml of 0.125 per cent sheep red blood cells sensitized with 20 units hemolysin were added. The mixtures were incubated at 37°C for 30 min. and then the cells were settle at 4°C for 30 min. The highest serum dilution showing 50 per cent hemolysis was taken as the CF titer.

9. *Hemagglutination inhibition test*

This test was carried out by a modification of Rosen's method (1960). Serial 2 fold dilution of antigens were prepared in 0.4 ml of PBS and 0.1 ml of 1 per cent rbc was added. Cells were settled at 37°C for 3 hours and the HA titer was expressed as the reciprocal of the highest dilution which showed one or two plus agglutination. For the HI test, 0.2 ml of 2 fold serially diluted serum was mixed with 4 units of HAnin in 0.2 ml and the mixtures were incubated at 37°C for one hours. 0.1 ml of 1 per cent rbc was added and tubes were reincubated at 37°C for 3 hours. The highest serum dilution showing complete inhibition of the agglutination was taken as the HI titer of the serum.

RESULTS

Relation between the three immunological tests in sera of patients



* The titers in parenthesis are for CF and HI

Fig. 1. Comparison of Three Serological Tests with Acute and Convalescent Sera Obtained from Three Children

The relation was examined using acute and convalescent sera obtained from three patients. Results are listed in Figs. la, b and c. Serum titers are expressed as the negative *log*, to the base 2, of the serum dilutions. As shown in the Figs., HI and NT antibodies appeared on the same day after the onset of the disease and gave almost the same titers. However, the CF antibody appeared a little later in one case and its titer was lower than those of the other two.

Comparison between the three immunological tests in sera of children

Sera were collected during a field trial of live vaccine from children under 5 years old who had neutralizing antibody to measles without evident history of this illness. They were tested by the three immunological techniques and the HI and CF titers found are summarized in relation to the NT titers (Figs. 2a and b). In this case, less than half the specimens showed detectable CF antibodies though the NT and HI antibodies were closely related. The geometric mean value ot the NT antibody titers was 7.0. That of the HI antibody titers was 6.2 and this was a little lower than that of former. In most cases NT and HI antibody titers were approximately proportional, but in a few cases, the HI antibody titers were remarkably lower than the NT antibody titers. It is not certain whether these special cases were valid observations or whether they were caused by technical errors.

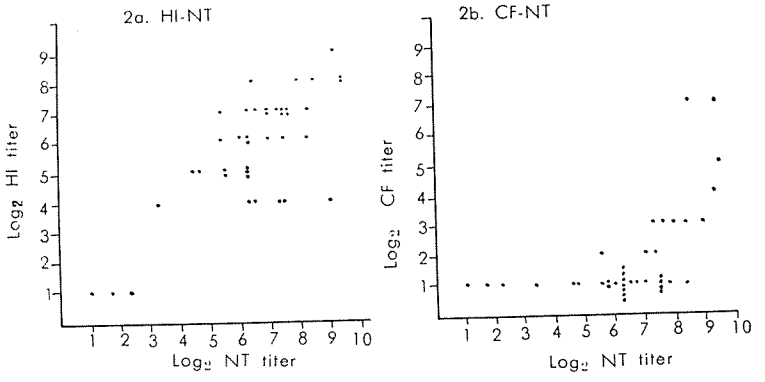


Fig. 2. Relationship between HI, CF and NT Antibody Tisters in Sero-Positive chidven Who Had No Previous History of Measles

Comparison of the three immunological tests with adult human sera and umbilical cord sera

Twenty one sera obtained from human adults and 22 sera from cord bloods were tested. The former were from people who had suffered from measles about 20 years ago.

All adult sera contained NT antibody though not at high level. These sera were tested for their HI titers after kaolin-monkey rbc treatment. The results are listed in Tables 1 and 2, together with the results of the CF test. Although the

NT and HI titers were well correlated, CF antibodies were hardly detectable with 2 units of antigen. After kaolin treatment of sera, the HI test was carried out at serum dilutions of above 1:16 and sera having 1:16 or lesser NT antibodies often did not inhibit hemagglutination.

The tests on cord sera gave similar results to those with adult human sera.

Table 1. Relationship between HI, NT and CF Antibody Titers in Human Adult Sera

Name	Age	Antibody Titers*		
		HI	NT	CF
Kitahara	21	4	4.5	<2
Oda	21	<4	3.75	<2
Shibutani	21	4	5.25	2
Soga	21	6	5.5	<2
Higaki	22	4	3.75	6
Kawamoto	23	4	3.0	2
Nakano	24	4	5.5	<2
Sakai	24	<4	3.67	<2
Uda	24	7	5.5	2
Matsushita	24	4	4.25	<2
Ikeda	24	4	4.5	2
Kitagawa	24	5	4.5	<2
Ueno	26	4	4.25	<2
Bando	26	6	—	<2
Kitaoka	26	5	5.25	<2
Nozaki	26	6	4.5	2
Shimazaki	27	5	4.25	2
Kobayashi	29	4	4.75	<2
Yoshida	29	4	4.0	<2
Hirose	32	<4	4.5	<2
Sato	35	<4	3.75	<2

* : Antibody titers are expressed as the reciprocal of $\log_2$

Serological responses of initially seronegative children to vaccination with live attenuated virus

Specimens converted from seronegative to seropositive during the field trial were selected for the comparison of the three serological tests. In this trials, three live vaccines, the Edmonston strain, Sugiyama strain and Toyoshima strain were employed with or without concomitant use of γ-globulin. First these 6 groups were treated separately, but no remarkable difference was observed between them with regard to their distributions and in their geometric mean titers. The results were, next treated as two groups: namely with and without γ-globulin injection, and the relations of the HI titer and CF titer to the NT titer were plotted (Figs. 3a and b, 4a and b).

Table 2. Relationship between HI, NT and CF Antibody Titers in Umbilical Cord Sera

No of Serum	Antibody Titers*		
	HI	NT	CF
1	<4	3.75	<2
2	4	4.25	<2
3	4	5.5	<2
4	<4	4.75	<2
5	5	4.5	2
6	5	—	<2
7	4	3.0	<2
8	5	4.5	<2
9	<4	3.75	<2
10	6	6.25	2
11	4	4.5	2
12	<4	3.75	<2
13	4	4.75	2
14	5	5.25	<2
15	4	4.75	<2
16	6	6.0	<2
17	3	4.5	3
18	4	4.75	2
19	3	3.75	2
20	5	4.75	<2
21	<4	2.75	<2
22	6	5.25	3

* Antibody titers are expressed as the reciprocal of $\log_2$

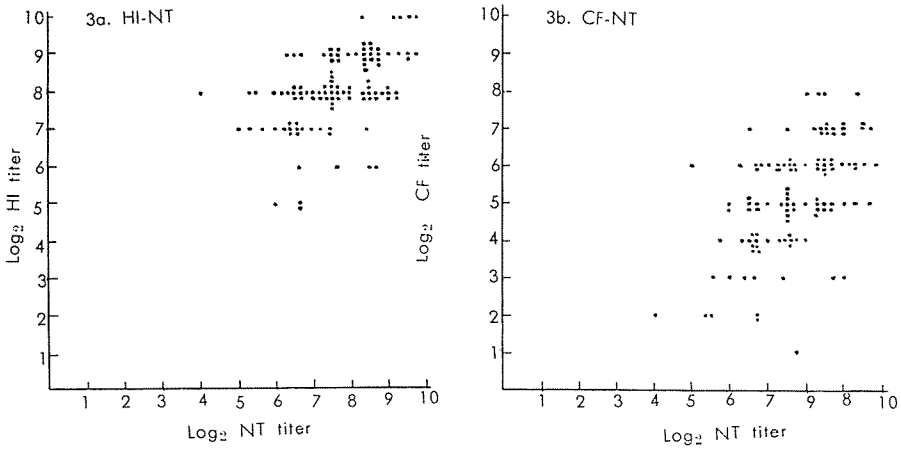


Fig. 3. Comparison of HI, CF and NT Antibody Levels after Live Vaccine Given

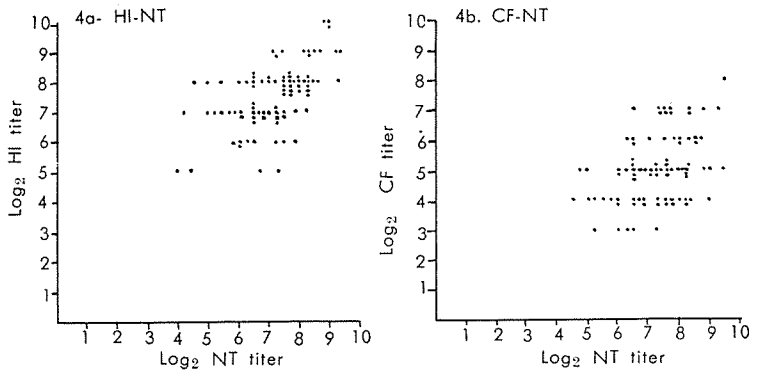


Fig. 4. Comparison of HI, CF and NT Antibody Levels after Live Vaccine Given with γ-Globulin

No great difference was found between these two groups. Thus the geometric mean titers of those inoculated with vaccine alone were 7.6 (NT), 8.1 (HI) and 5.2 (CF), and those of inoculated vaccine and γ-globulin were 7.1, 7.6 and 4.9 respectively. The titers of NT and HI antibodies again were well correlated. Although most sera gave a positive CF reaction, the antibody titers were considerably lower than those in the two other tests and a few specimens did not react with 2 units of CF antigen.

Recognition of the booster effect of live attenuated measles vaccine

In the field trial, 40 cases had antibodies before vaccination. Thirty two of these cases were found in vaccinated and 8 in placebo groups. Nine children out

Table 3. Serological and Clincal Response in Seropositive Children One Month after Vaccination with Live Measles Vaccine

No of Children	Age of Children	Antibody Titers*						Clinical symptoms
		Pre-Vaccination			Post-Vaccination			
		CF	HI	NT	CF	HI	NT	
1G-304	2yrs. 2mos.	<2	4	3.33	<2	6	7.0	maximum fever 37.2°C on 8th and 9th days, little rash on face
1M-2	3. 8.	<2	5	4.5	<2	7	8.0	no symptoms
2M-6	3. 2.	<2	3	1.0	<2	6	5.67	no symptoms
4G-133	2. 1.	3	4	9.0	7	10	11.0	fever (maximum 38.0°C on 22nd day)
4G-221	4. 3.	<2	5	6.33	2	7	8.5	no symptoms
4G-333	1. 9.	<2	7	7.5	3	8	9.33	no symptoms
4G-042	5.	3	7	8.33	7	8	6.5	no symptoms
5G-241	1.	<2	5	6.33	2	8	7.5	no symptoms
7G-386	4. 3.	<2	8	6.5	<2	8	9.33	no symptoms

* : Antibody titers are expressed as the reciprocal of $\log_2$

of the 32 showed a serological response to vaccination whereas none of the 8 placebo cases did. Results of serological tests on paired sera from these cases are listed in Table 3 together with brief notes on the clinical reactions. Seven cases showed no clinical reaction and the remaining 2 exhibited only dubious clinical symptoms. In these cases, serological responses could be recognized by both the NT and HI tests, but rarely by the CF test.

DISCUSSION

This report brings up many technical problems, since efforts were made to solve difficulties in the application of measles serological tests. Techniques for neutralization and complement fixation tests employed were adopted by the Measles Vaccine Research Association of Japan from a rather practical reasons. For example, the neutralization end point was determined on the day when marked cytopathic changes appeared in all 5 tubes inoculated with a 1/10 dose of the virus used for neutralization, though the virus titer was determined by 14 days

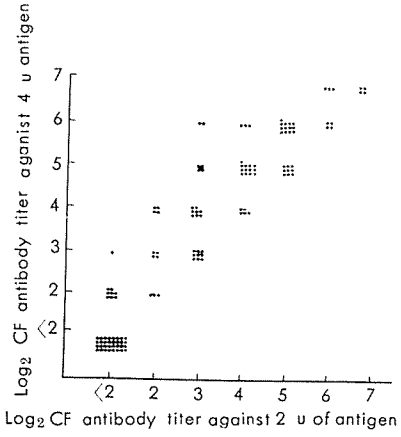


Fig. 5. Effect of 2 and 4 Units of CF Antigen in Test on Antibody Titer of Sera

observation. To maintain the reproducibility and rapidity of the test, 1 to 2 day old FL cultures were employed. When old cultures were employed, not only was the appearance of cytopathic changes greatly delayed, but accurate microscopic tests were difficult. Changing the medium accelerated the appearance of cytopathic changes and made observations easier. With regard to the CF test, difficulties in obtaining CF antigen of high titer forced us to adopt tests using 2 units of antigens (Meyer *et al.*, 1962). However, it would be better to use 4 units of CF antigen since the sensitivity of the CF test increased considerably when 4 rather than 2 units of antigen were used (Fig. 5).

In our laboratory, the antigen was prepared from infected FL cultures after disrupting the cells by sonication and it contained 8-32 units of CF antigen in 0.1 ml. This antigen was as specific as those prepared by ordinary methods (freezing -thawing) and showed little or no anticomplemental activity. The first problem in the HI test arose when HAnin was employed. It was prepared from infected KB cells by centrifugal concentration and Tween 80-ether treatment. In this procedure, KB cells were used since more HAnin was produced in this culture than in FL cultures and Tween 80-ether treatment was employed in order to disperse sedimented HAnin and for partial purification. The HAnin titer was increased by this treatment and the sensitivity of the HI test also increased when this antigen was employed (Norrby, 1962; Funahashi and kitawaki, 1963). The specificity of this antigen had been established by Norrby (1962) and by Funahashi and kitawaki (1963). The second problem was with regard to the rbc used. Standard antigen was distributed in tubes in about 1 ml aliquots and stored at -20°C. Monkey rbc were tested by this antigen and selected for the HI test. The storage conditions of standard antigen was shown by Norrby to be unsuitable for this antigen. These conditions were employed here to maintain the sterility of the antigen during the preparation and because under these conditions activity was retained for several months. The third problem involved the treatment of the sera for the HI test. Nonspecific inhibitor was removed by kaolin absorption and serum hemagglutinin to monkey rbcs was removed by absorption with monkey rbcs. Nonspecific inhibitor was also removed by washing the preparation with cold acetone. For the HI test at dilutions of under 1:16, this treatment was employed, combined with absorption with packed monkey rbc. At higher serum concentrations used, agglutinated rbc were sometimes sedimented, as if the agglutination was completely inhibited. Although this apparent inhibition could be distignuished from real inhibition by the patterns of the sedimented cells, the differentiation was readily made when cells were reshaken after the first reading and then resettled for 30-60 min.

The sensitivity and reliability of the HI test was first tested with sera from patients with acute measles and those in the convalescent stage. HI antibody appeared and increased in the same way as NT antibody. This close relation suggests that the HI test is suitable for routine tests or large scale trials and it avoids the complexity and difficulties of the NT test for measles. The relation of the HI and NT tests was examined further with sera of subjects in different conditions. Sera from children of under school age were tested in the same way as samples from subjects who had had measles a few years previously. Unexpectedly many sera which neutralized the virus showed a CF titer of less than 1:4 the HI antibody titer was closely related to the NT antibody titer. This correlation was examined with sera of human adults who had had measles 20-30 years previously and with sera from umbilical cord sera. The CF test using 2 units of antigen is

unsuitable for detection of measles antibody in these cases, but the correlation between the NT and HI antibodies were again confirmed in the two types of sera.

The HI test was first applied to serum in field trials on live vaccines arranged and carried out by the Measles Vaccine Research Association in accordance with the WHO plan. Paired sera from children who were initially sero-negative were tested. A correlation between NT and HI antibodies were again confirmed. The levels of NT antibodies were approximately proportional, but the factors responsible for the difference in the NT/HI ratio in various samples is unknown. The CF test is regarded as reliable for tests on the sera of subjects in the convalescent stage or those who have recently been vaccinated (Black, 1959; Bech, 1959). However, five initially sero-negative children had both NT and HI antibodies after vaccination though they did not have CF antibody. Four of these sera were obtained from children who were concomitantly injected with γ-globulin. Such particular cases had not been observed in our laboratory and they might possibly have been due to the effect of γ-globulin injection. The other case was found in a group inoculated by the inhalation method. This case is most likely explained by assuming that the serum contained as little as less than 1:2 of antibody before vaccination and that this affected the response to CF antibody. Trials were conducted to compare vaccines developed in three different laboratories (Okuno *et al.*, 1960; Enders *et al.*, 1960; Matsumoto *et al.*, 1962). The distribution of serum titers on serologically converted sera on three antibodies and the geometric mean titers of each antibody did not differ greatly from each other in these three vaccines.

Fourty children of the 344 tested had antibody before vaccination though they had no evident history of measles. Thirty two of these were in the vaccinated group and 8 were in the placebo group. None of the placebo group showed antibody response, but 9 of the 32 vaccinated subjects showed a serological response to vaccination. None of these children exhibited any remarkable clinical recation, though one of them had fever on the 22nd day after vaccination and another had a temperature of 37.0°-37.2°C on the 8th and 9th days after infection. These cases may be explained by the booster effect of vaccination like those recognized by Stockes *et al.* (1962) in natural measles and by Matsumoto *et al.* (1962) after vaccination with attenuated virus. Such responses could be detected by the HI test as well as the NT test, but hardly ever by the CF test.

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