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THE USE OF MICE IN POTENCY ASSAYS OF INACTIVATED MEASLES VIRUS VACCINES*

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SUMMARY Mice responded well to intraperitoneal inoculation of measles antigen and a single intraperitoneal injection of antigen in mice can be employed as an highly reproducible method for potency assay of inactivated vaccine. The antigen extinction limit values determined by the mouse potency test were proportional to those determined by the guinea pig test. These values did not seem to be affected by whether the vaccine was plain or adsorbed to alum or to aluminum phosphate, but seemed to reflect the total dose of antigen. Subcutaneous injection of antigen in mice resulted in a good antibody response to adsorbed vaccine, but a poor response to plain vaccine.

INTRODUCTION

The development of an efficient immunization method against measles in which inactivated vaccine was given prior to the administration of live vaccine, resulted in an increased demand for inactivated vaccine. A reproducible and economical method for potency assays was necessary so that the vaccination schedules for administration of the combination of killed and live vaccines could be improved.

GARD *et al.* developed an accurate procedure for assay of the potency of inactivated poliovirus vaccines using guinea pigs (1956). This methos has been applied for assay of the antigenicity of measles virus by many workers (DEWITT and NOOK, 1960; HILLEMAN *et al.*, 1962; WARREN *et al.*, 1962). The sensitivities of rabbits and chickens have also been tested (DEWITT and NOOK, 1960; WARREN *et al.*, 1962), but it was not clear whether they offer any advantages over guinea pigs in the potency test. Mouse potency test seemes to meet economical requirements in the assay of the potency of inactivated influenza virus vaccine (NISHIKAWA *et al.*, 1953).

This report deals with the sensitivity and accuracy of potency assays of measles virus vaccine using mice.

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MATERIALS AND METHODS

1. *Virus*

The Sugiyama strain was employed for neutralization tests. The Toyoshima strain was employed for hemagglutination inhibition (HI) tests and for preparation of reference vaccines.

2. *Vaccines*

Reference vaccine for potency assays was prepared from infected tissue culture fluid of FL cultures maintained in medium 199 without serum. The supernatant fluids, containing 10^5–10^6 TCD_{50} virus per 0.1 ml, were mixed with formalin at a final concentration of 1 : 3000 and incubated at 4°C. When inactivation was complete, the materials were stored in test tubes at −20°C. Killed vaccines for practical use were prepared in the Research Foundation for Microbial Diseases of Osaka University from monkey kidney tissue cultures infected with the Tanabe strain (MUTAI, 1959) of measles virus.

3. *Animals*

Four week old mice of the dd strain (Shizuoka farm) and guinea pigs, weighing 250–270 g, were employed for the potency tests.

4. *Serological tests*

Antibodies to measles were determined by the neutralization (ordinary method) and HI (micro HI technique) tests according to the method described previously (TOYOSHIMA *et al.*, 1965).

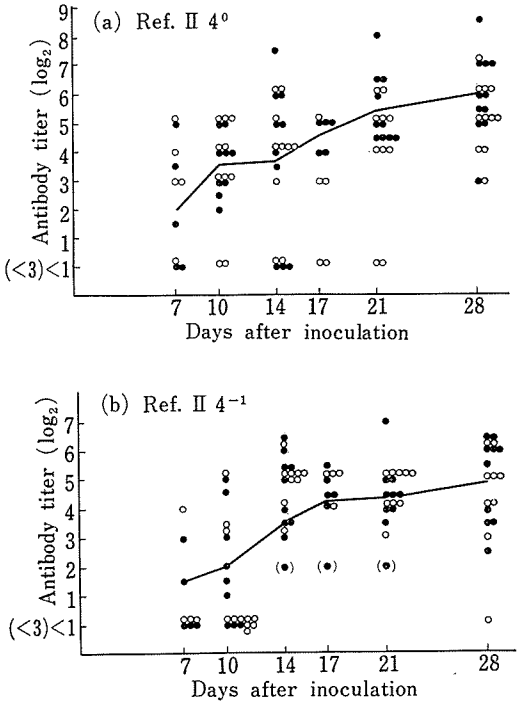
5. *Antigen extinction limit (AEL)*

Four-fold serial dilutions of vaccines were made in phosphate buffered saline. Groups of 10 mice or 5 guinea pigs were immunized according to appropriate schedules and the animals were bled by heart puncture at the end of these immunization schedules. Undiluted (guinea pigs), or 2-fold diluted (mice), sera from each animal was heated at 56°C for 30 min. and then its neutralizing antibody was measured. The HI antibody of sera treated with kaolin and monkey erythrocytes were tested (lowest dilution of serum, 1 : 8). The antigen extinction limits (ED_{50}) were calculated by the Read and Muench formula as the highest dilution which gave a measurable antibody response in 50 per cent of the animals either by the neutralization or the HI test.

RESULTS

1. *Antibody response of mice to intraperitoneal injection of formalin-inactivated measles virus*

The antibody response of mice to immunization with formalin-inactivated plain vaccines were measured to find the optimum intervals between intraperitoneal injection of antigen and bleeding. Serial 4-fold dilutions of reference II vaccine were made in phosphate buffered saline. Fifty to 52 mice were inoculated intraperitoneally with 0.5 ml of each dilution and 5–12 mice were bled at appropriate intervals of between 7 and 28 days after inoculation. The neutralizing and HI antibodies were tested with 2-fold serial dilutions of individual sera. Figs. 1a, b, c, and d showed the antibody titers of each animal and the geometric mean of their neutralizing antibody titers. The geometric mean antibody level increased gradually for 28 days after inoculation with the two lower dilutions of vaccine (Figs. la and b), but reached a maximum after 21 days with the two higher



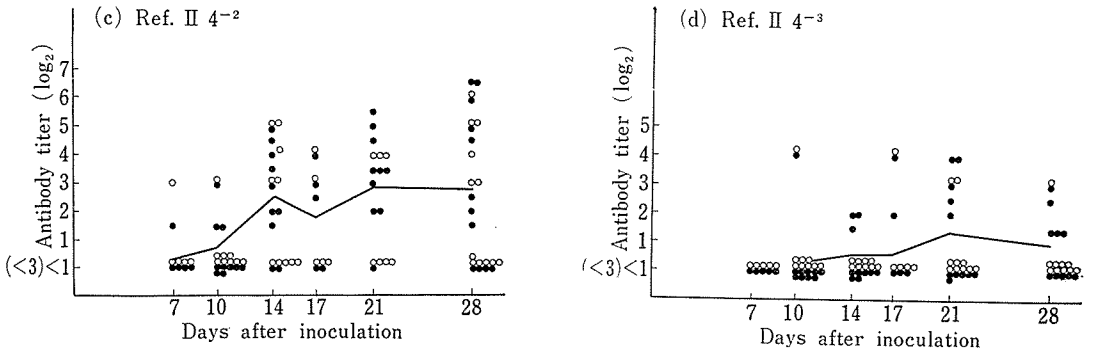


FIGURE 1a, b, c, d Response of mice inoculated with formalin-inactivated measles virus via intraperitoneal route
——— Geometric mean of neutralizing antibody titer
● Neutralization ○ Hemagglutination inhibition

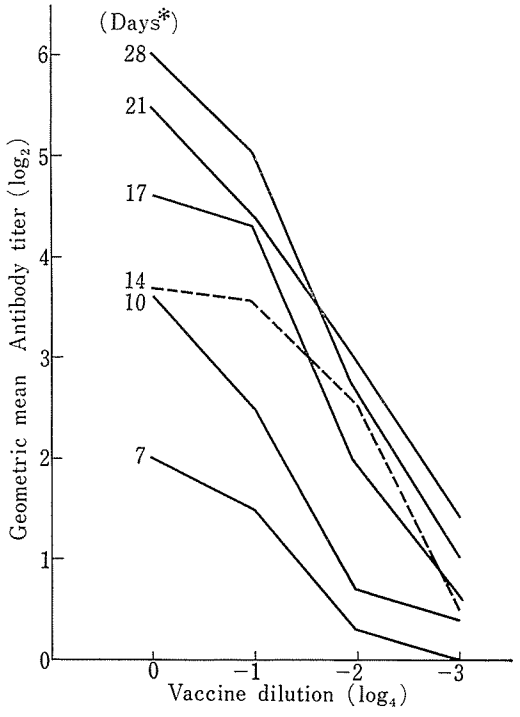


FIGURE 2 Dose response of mice to plain vaccine with various intervals between times of inoculation and bleeding
* Days after intraperitoneal injection

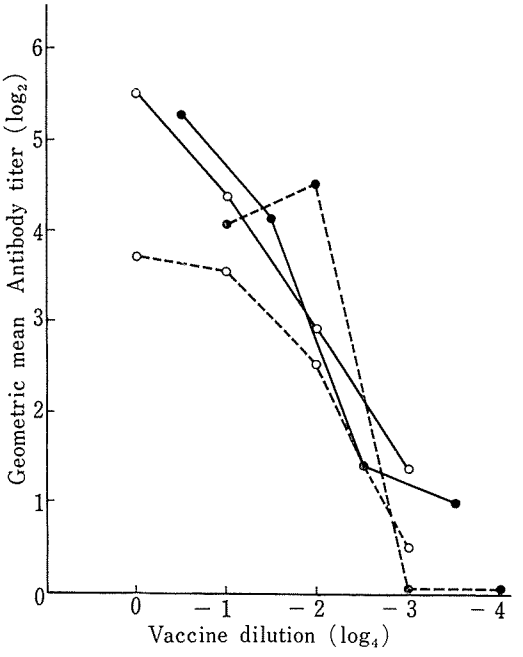


FIGURE 3 Dose response of mice to plain and adsorbed vaccines
○——○ Plain 3 weeks after intraperitoneal injection of vaccine
●——● Adsorbed 3 weeks
○------○ Plain 2 weeks
●------● Adsorbed 2 weeks

dilutions (Figs. 1c and d). The geometric mean antibody titers were plotted against the vaccine dilution at each bleeding time (Fig. 2). Quite high antibody evels were obtained 14 days after inoculation even with diluted vaccines and almost linear relations of antibody level (log_2) to vaccine dilution (log_4) were observed 21 and 28 days after inoc-

ulations. The highest antibody response was obtained 28 days after inoculation with undiluted vaccine, but the slope was steeper on the 28th day than on the 21st day and the antibody levels were reversed between 4- and 16-fold dilution of vaccine. As shown in Fig. 3, aluminum phosphate adsorbed vaccine gave a similar dose response curve to that of plain vaccine 21 days after inoculation. These results suggest that when a single intraperitoneal injection is given, 21 days is the optimum interval between inoculation and bleeding for assays of potency in mice.

2. *The sensitivity and reproducibility of the antigen extinction limit (AEL) method for potency test in mice*

The AEL values were determined by both the neutralization and HI tests. As shown in Fig. 4, the values in the neutralization test increased rapidly for 14 days after inoculation and then gradually increased to the maximum after 21 days. The values dtermined by HI were less sensitive and the curves were rather irregular. The reproducibility of the test was examined with 2 plain vaccines (Table 1). Reference I was tissue culture fluid concentrated 4-fold by ammonium sulfate precipitation (KITAWAKI *et al.*, 1964) after inactivation of the measles virus. A three week interval between inoculation and bleeding were employed as standard in the " 3 week test " and a 2 week interval was also tested to see if it was possible in practise to shorten the test period in the " 2 week test ". The " 3 week test " was slightly more sensitive than the " 2 week test ", but the reproducibility of the results were quite good in both, except that two extremely low AEL values were obtained in two " 3 week tests " which was surprising considering the reproducibility of the other results. Eight of 9 other vaccines, tested at the same time as these 2, showed similar low AEL values, although exceptional case showed a value which was almost equal to the mean AEL value in

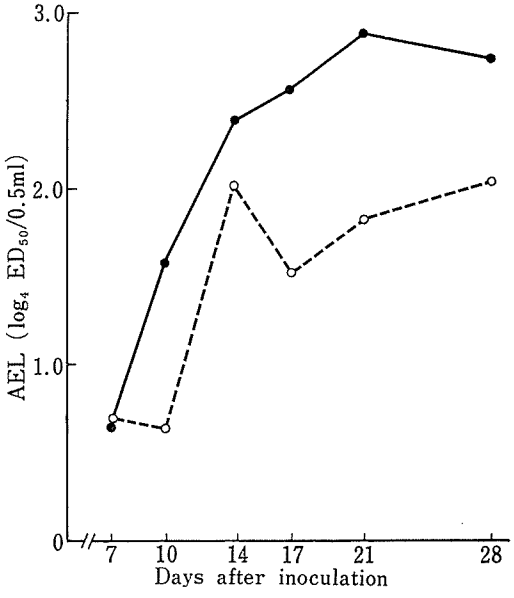


FIGURE 4 Change of antigen extinction limit (ED_{50}) with interval between times of inoculation and bleeding

●——● Neutralization

○-----○ Hemagglutination inhibition

TABLE 1 *Reproducibility of potency test using mice*

Vacc.	Ref. 1 2 wks.		Ref. 2 2 wks.		Ref. 2 3 wks.	
	Neut.	HI	Neut.	HI	Neut.	HI
AEL (ED_{50} per 0.5 ml)		2.4	2.4	1.5	2.6	1.8
		2.1	2.2	2.0	2.8	1.8
		2.7	2.3	1.5	3.0	
		2.3	2.3	1.7	2.6	
	3.1	2.7	2.0	1.4	1.7*	
	3.2	2.2	2.4	2.0	2.2	1.9
	3.2	2.2	2.0	1.0	1.7*	1.2
					2.3	
					2.2	
					2.3	
					2.9	
					2.9	
GM ±SD	3.17 ±0.05	2.37 ±0.23	2.28 ±0.15	1.62 ±0.21	2.43 ±0.42	1.68 ±0.28

* Special cases, discussed in the text

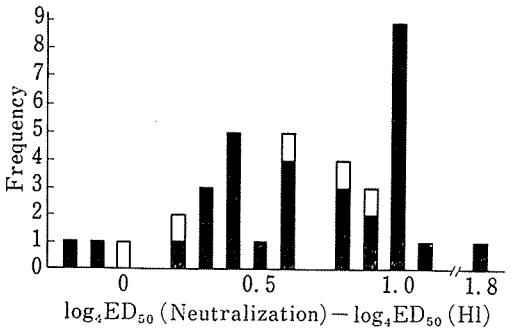


FIGURE 5 Relation between AEL values calculated from neutralization and HI tests
□ Guinea pig ED_{50} ■ Mouse ED_{50}

the other experiments. The AEL values calculated from the HI test seemed to be reproducible (Table 1), but not to be directly correlated with the values calculated from the neutralization test (Fig. 5).

3. *Effect of single and multiple injections on the AEL value*

The effect of multiple intraperitoneal injections of plain and aluminum phosphate adsorbed vaccines on the AEL value was tested following the schedule shown in Table 2. With plain vaccines, the AEL values increased with increase in the vaccine dose and with increase in the number of injections given when the total dose was constant, but no striking difference in the AEL values was observed with adsorbed vaccine.

TABLE 2 *Effect of multiple injections on AEL values*

Vaccine \ Immunization schedule	0*~14**	0.7~14	0.3.7~14
Ref. 1	3.2***	3.6	4.8
Ref. 1 (2^{-1})	2.8	3.6	
Ref. 1 (3^{-1})	ND	ND	3.9
Vaccine \ Immunization schedule	**0~21**	**0.7~21**	
4 A (plain)	1.4	2.6	
〃	1.5	3.1	
4 C (adsorbed)	2.6	2.8	
〃	2.7	2.7	

* Date of inoculation ** Date of bleeding
*** ED_{50}/0.5 ml

4. *Comparison of AEL values in the mouse and guinea pig potency tests*

To measure the correlation of the mouse potency test and the guinea pig potency test, experiments were carried out simultaneously with 7 plain and 2 adsorbed vaccines. Mice were inoculated intraperiotoneally with 0.5 ml of serially diluted antigens and bled 3 weeks after the inoculation. Guinea pigs were inoculated subcutaneously with 1.0 ml of antigen twice with a week's interval between injections and bled 3 weeks after the first inoculation. AEL values were calculated from the neutralization test, as described in the MATERIALS AND METHODS section. An almost linear relation was observed between these 2 potency tests (Fig. 6), although the sensitivity of the test in mice was about $4^{1.8}$-fold lower than that in guinea pigs.

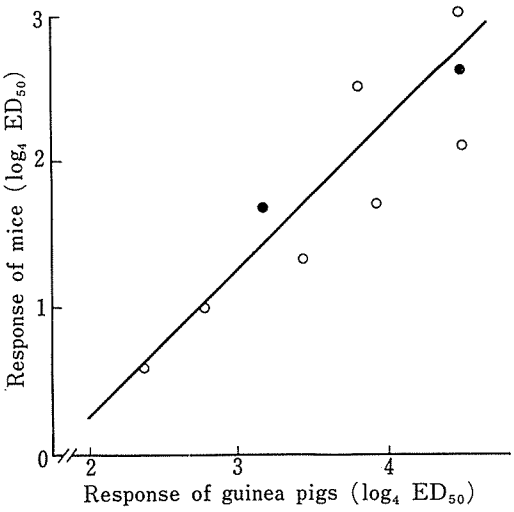


FIGURE 6 Comparison of mouse- and guinea pig-potency tests
● Plain ○ Adsorbed

5. *Comparison of the antibody response to injection of plain and adsorbed vaccines via different routes*

Antibody levels induced by adsorbed vaccines seems to be somewhat higher than those induced by plain vaccines. Therefore three different type vaccines were tested for their capacities to induce an antibody response in mice. An inactivated vaccine sample was divided into three parts. One part was adsorbed by aluminum phosphate (5 mg/ml) and one by alum (10 mg/ml), while the third was not treated. Their AEL titers were measured by the " 3 week test ". A further test on these vaccines by the subcutaneous route was performed simultaneously. The AEL values of the three vaccines were essentially the same when the mice were inoculated intraperitoneally. When vaccines were inoculated subcutaneously, the AEL value of the plain vaccine was quite low, although the two adsorbed vaccines showed similar AEL values to those determined by intraperitoneal injection (Table 3). The neutralizing antibody titers were determined with pooled sera of each group of mice and the geometric mean titers of duplicate tests were plotted against the vaccine dilution (Figs. 7 and 8). The antibody levels of mice inoculated intraperitoneally with adsorbed vaccines were roughly the same but were $2^{1.5}$–$2^{2.0}$-fold higher than those with plain vaccine. The antibody response to plain vaccine by the subcutaneous route was much lower than to the vaccine by the intraperitoneal route. However, the two adsorbed vaccines produced similar antibody responses by subcutaneous injection and by intraperitoneal injection.

TABLE 3 *Comparison of intraperitioneal and subcutaneous routes of inoculation for potency test*

Vaccine \ Route	Intraperitoneal		Subcutaneous	
Exp.	1	2	1	2
Plain	2.6(2.57)	2.3(2.33)	0.1	<0
$AlPO_4$	2.6(2.56)	2.4(2.38)	2.3	2.2(2.24)
Alumn	2.6(2.58)	2.4(2.42)	ND	2.2(2.20)

Numbers : ED_{50}/0.5 ml

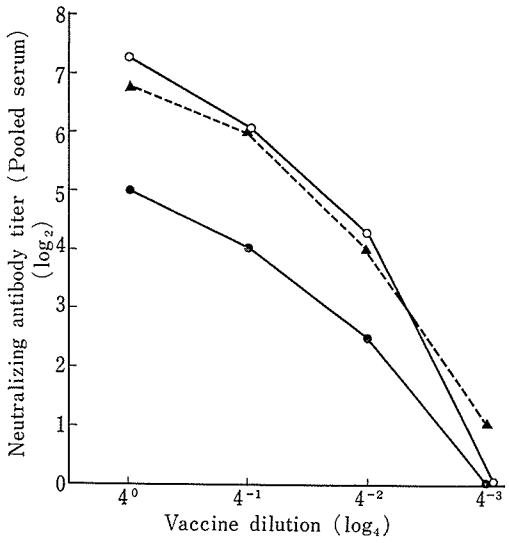


FIGURE 7 Antibody response of mice to intraperitoneal inoculation of various vaccines
○———○ Alum adsorbed
▲------▲ $AlPO_2$ adsorbed
●———● Aqueous

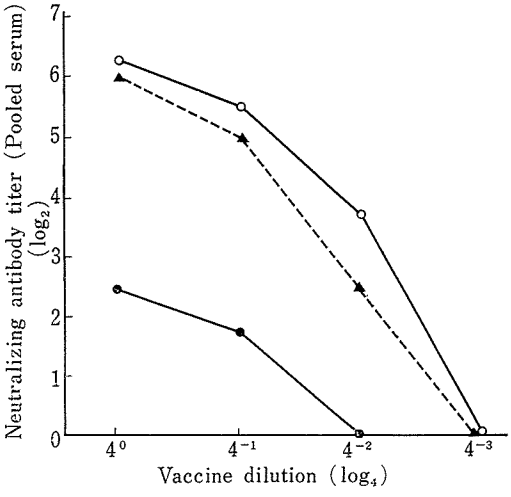


FIGURE 8 Antibody response of mice to subcutaneous inoculation of various vaccines
○———○ Alum adsorbed
▲------▲ $AlPO_2$ adsorbed
●———● Aqueous

DISCUSSION

The requirement for inactivated measles virus vaccine has been increased by the use of inactivated vaccine prior to the administration of live vaccine. Although guinea pigs were employed for assay of the potency of this vaccine (DEWITT and NOOK, 1960; HILLEMAN *et al.*, 1962; WARREN *et al.*, 1962), large numbers of guinea pigs are not readily available in most laboratories in Japan. Therefore, the possibility of using mice for assay of the potency of measles vaccines was examined.

The mice (dd strain) responded well to a single intraperitoneal injection of measles antigen. The antibody levels of mice inoculated with a high antigen dose increased steadily during the 28 day observation period. However, the levels in animals inoculated with diluted antigens reached a maximum 21 days after the inoculation and the antibody response to the vaccine dose was linear on the 21st day. The antigen extinction limits were calculated as the reciprocal of the vaccine dilution which produced a neutralizing antibody response (1 : 2 or more) in 50 per cent of the inoculated animals. Maximum AEL values were obtained 21 days after inoculation with aqueous vaccine. From these results, the reproducibility of the test was examined with plain vaccine by the " 3 week test ". The possibility of using a " 2 week test " was also examined to see whether the observation period could be shortened. Reproducibility of the test was fairly good in both tests but two exceptionally low AEL values were obtained by the " 3 week test ". Eight of the 9 vaccines tested simultaneously on the same batches of mice showed much lower AEL values than those tested with other batches of mice. The remaining vaccine showed similar AEL values in the test to those with other batches of mice. This difference in response of mice was only seen with these 2 batches, and may have been caused by some accident, possibly infection, during breeding, transportation or immunization though this has not been confirmed. The potency values of vaccine for practical use may be determined by the mouse potency test and the values can be standardized with the AEL values of reference vaccine tested at the same time with the same batches of mice. When the AEL values of the reference vaccines are extraordinary, i.e. when they show inclinations of over $4^{0.5}$ from the geometric mean of other experiments, all the results obtained using the same batch of mice should be rejected.

This test was performed simultaneously with the guinea pig test and the two were found to be proportional. However, the sensitivity of the mouse test was $2^{1.7}$-fold less than that of guinea pig test. The sensitivity could be raised by the multiple injection method with plain vaccines, but not with adsorbed vaccine within the 21 day observation period. The AEL values determined by a single intraperitoneal injection into mice seemed to indicate the quantity of antigen in the vaccine and not to indicate directly the efficacy of the vaccine for practical use. A comparative study on the sensitivities of children to inoculation of vaccine and the AEL values is required for establishing the applicability of this test. Table 3, Figs. 7 and 8 suggest that different standards are required at least for vaccines of different compositions, i.e. adsorbed, plain, and disrupted particles. The relations between the antibody response of humans and the composition of the vaccine will be reported in the near future.

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