Assay of neutralizing antibody against variola virus by the degree of focus reduction on HeLa cell cultures and its application to revaccination with smallpox vaccines of various potencies

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A method for assaying neutralizing antibody against variola virus was established by focus counting on HeLa cell cultures. The ND_{50} titre, i.e., the serum dilution endpoint to give a 50% reduction in the number of foci, was determined with excellent reproducibility.

Groups of students 19–20 years of age were revaccinated by the multiple pressure method with serial 10-fold dilutions of a smallpox vaccine and their neutralizing antibody response was assayed by the focus counting assay system and was related to the local skin reactions on the seventh day after inoculation and to the potency of the vaccine administered. There was a significant rise in the antibody level even after inoculation with a vaccine whose potency was as low as 1.3×10^5 pock-forming units/ml. In general, the rise in the log antibody level was proportional to the diameter of the reddening, but a significant rise was found among individuals who had no detectable skin reaction. The skin reaction was greater among individuals with a lower initial antibody level when the vaccine administered had a potency lower than 1.3×10^6 pock-forming units/ml.

Procedures for the titration of neutralizing antibody against poxviruses have been described with various host cell–challenge virus systems. In most cases, the reduction of vaccinia virus infectivity was assayed on chick chorioallantoic membranes (CAM) (Boulter, 1957; McCarthy et al., 1958a, 1958b), primary monkey kidney cell (Cutchins et al., 1960; Middlehoven, 1962), primary chick embryo cell (Andersen & Larsen, 1966), or on established line, HeLa cell cultures (Kitamura et al., 1964) by measuring focal lesions or virus-induced enzyme activity in the human amnion cell line, AV_3, made deficient of normal enzyme activity (Ziegler & Hutchinson, 1969). The neutralization test with variola virus is important as it provides a way of defining immunity against smallpox after smallpox vaccination, but attempts to use it have been restricted to the pock reduction method on CAM (Downie et al., 1961; Anderson & Skegg, 1970), possibly because of the lack of a quantitative *in vitro* assay method. Anderson & Skegg (1970) carried out a collaborative study to compare the neutralizing antibody titre of convalescent serum from a smallpox patient using various challenge virus–host cell systems and concluded that better agreement between laboratories could be obtained with the pock reduction method than with the plaque reduction method.

Kitamura (1968) has established a quantitative macroscopic method of assaying variola virus infectivity *in vitro* by modifying the hyperplastic microfocus counting method on HeLa cell cultures (Pirsch & Purlson, 1962); this allows the quantity of variola virus to be determined with an ease and accuracy comparable to that found with plaque counts of vaccinia and other animal viruses. In the first part of the present paper the application of this focus counting method to the estimation of 50% neutralization titres (ND_{50}) against variola virus is reported. In the second part, the antibody response to revaccination with smallpox vaccines measured with the procedure developed here is analysed with special reference to

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several disputable points concerning revaccination —e.g., the correlation between the neutralizing antibody level at the time of vaccination and the local skin reaction (Esmark, 1965), the relation between the severity of the local reaction and the rise of the neutralizing antibody (Kitamura et al., 1964), and the effect of equivocal reactions on the antibody levels.

MATERIALS AND METHODS

Virus

Variola major virus, strain Harvey (VHE), was used as an infected CAM homogenate after two or three passages through CAM.

Virus titration on HeLa cell cultures

HeLa cell cultures in 4.5-cm glass Petri dishes and virus titration by counting foci on them have already been described (Kitamura, 1968). The pock counts were made after inoculating 0.1 ml of the virus preparation on to the CAM of 12-day-old chick embryos and incubation at 37°C for 3 days.

Antibody preparations

Normal rabbit serum (NRS) was obtained from healthy rabbits that had no antibodies against poxviruses, detectable by the following tests: complement fixation, haemagglutination inhibition, neutralization (variola pock reduction), agar gel precipitation, and indirect fluorescent staining. Standard immune rabbit serum (VcIRS) was prepared by inoculating about 10^8 pock-forming units of dermovaccinia virus, strain Dairen-I (DIE), after 4 passages through rabbit kidney cells, on to the scarified skin of female rabbits and bleeding them 2 weeks after an intravenous booster inoculation in the 6th week of infection. Antivariola rabbit serum was prepared by repeated intravenous injections of VHE virion suspension, of CAM origin, that had been partially purified by two cycles of fluorocarbon treatment and differential centrifugation (Tagaya et al., 1963), and that contained about 10^6 pock-forming units per dose. The International Standard for Anti-Smallpox Serum (WIS), supplied by the World Health Organization, contained 1 000 International Units (IU) per ml of neutralizing antibody (Anderson, 1965). Vaccinia immune gamma-globulin (VIG) was supplied by Dr S. Ishimori, Japanese Red Cross Blood Centre, Tokyo, as an 8.4% (w/v) solution of the IgG fraction from pooled human plasma of recently revaccinated adults prepared by Cohn's ethanol procedure.

Neutralization test

The antibody preparations, which were heated at 56°C for 30 min before assay, and the challenge virus were diluted with a mixture of equal volumes of Hanks' BSS and phosphate-buffered saline, pH 7.6 (HP). A 1-ml aliquot of 2- or 4-fold serial dilutions of the antibody preparation was mixed with an equal volume of VHE virus and incubated at 37°C for 60 min and at 4°C overnight. ND_{50} titres were determined as the serum dilution that gave a 50% reduction in the number of focal lesions, pocks on CAM, or foci on HeLa cell plates, compared with the number formed by the same dilution of VHE virus with NRS, by plotting the results on semilogarithmic paper. Complement was not added to the reaction mixture for the reasons explained in the Discussion section of this paper. The plaque-reduction assays with the vaccinia virus (DIE) were carried out as described previously (Kitamura et al., 1964).

Human revaccination experiment

A calf-lymph type vaccine with a potency of 1.3×10^8 pock-forming units/ml[1] was diluted serially 10-fold with HP to 10^{-1}, 10^{-2}, and 10^{-3} immediately before the inoculation. Groups of about 30 female college students, 19–20 years of age, all of whom had had their last smallpox vaccination at least 8 years previously, were inoculated with one of the vaccine dilutions by putting 0.01 ml of the inoculum within a circular region of 6-mm diameter on the extensor side of the left upper arm, followed by 20 pressures with a vaccination needle. Skin reactions were read 7 days later and the general reactions, recorded daily by the subjects themselves, were noted at the same time. Serum preparations were collected just before vaccination and 6 weeks after vaccination, and neutralizing antibody titres were determined after heat inactivation at 56°C for 30 min.

The skin reactions were described using the following criteria: + reaction, reddening of more than 8-mm diameter with the formation of a vaccinal vesicle or pustule; ± reaction, reddening of more than 8-mm diameter with palpable induration; – reaction, reddening without induration or no reaction. The + and ± reactions are those classified as " major reactions " by the WHO Expert Committee on Smallpox (1964) whereas the – reaction was classified by the Committee as an " equivocal reaction ".

[1] Manufactured using the Ikeda strain of vaccinia virus supplied by the Kitasato Institute, Tokyo.

RESULTS

Characterization of the assay procedure

The neutralization curve with different antibody preparations. Standard immune rabbit sera against vaccinia (VcIRS) and variola viruses (VrIRS) and the serum from a human adult revaccinated 12 years earlier were serially diluted 10-fold and submitted to the neutralization test following the procedure described above. The numbers of foci in control plates (inoculated with challenge virus mixed with diluted NRS) were almost constant irrespective of the dilution used and an average value of 324 foci per plate was used as a 100% survival value. Results are shown in Fig. 1 (top). Both VcIRS and VrIRS showed typical sigmoid curves with a steep, straight portion at around 50% neutralization, as observed in the case of the vaccinia plaque reduction method (Kitamura et al., 1964). Serum from an adult vaccinated 12 years earlier gave a similar and parallel curve. In the second experiment, antibody preparations from human sera were assayed in the same way with 4-fold serial dilutions. The results of this experiment, also given in Fig. 1, show that the International Standard for Anti-Smallpox Serum (WIS) gave a neutralizing antibody titre identical to that of the immune rabbit sera depicted in Fig. 1 (top). Serum pools from recently revaccinated adults and VIG (IgG fraction) from a pool of such sera also gave similar sigmoid curves, with the straight portions of the curves practically parallel. Thus with all the materials tested, it was easy to determine the dilution of antibody preparation that gave 50% neutralization without significant ambiguity, by simply connecting the plotted points by hand. The 50% neutralizing dose (ND_{50}) is defined here as the rate of antibody dilution that, after mixing with an equal volume of the challenge virus, causes a 50% reduction in the number of foci. In a few trial experiments, there was no significant modification of the sigmoid curve by extending the time of incubation at 37°C beyond 40 min. Shorter incubation resulted in irregularities in the sigmoid curves and the curves were shifted towards the lower end of the dilution scale, suggesting that 40 min of incubation was necessary for saturation at 37°C. Omission of the incubation at 4°C gave a slightly lower ND_{50} titre, possibly as a result of a higher rate of survival at the lower dilutions of the serum, suggesting that neutralization consists of two different mechanisms that proceed at 37°C and 4°C, respectively. There was no significant difference between the ND_{50} titres obtained by comparative assays using HP, YLE (Earle's saline with 0.1% yeast extract and 0.5% lactalbumin hydrolysate), or Eagle's MEM as the diluent, but the use of simple PBS gave a rather irregular sigmoid curve, possibly as a result of aggregation of the challenge virus before the reaction with the antibody.

Reproducibility of the assay. Two preparations of human serum, WIS and serum from a revaccinated human male adult, were diluted 1 : 10 with HP and stored in a deep-freeze unit at -25°C after being dispensed into test tubes. The ND_{50} titre of an aliquot from the two preparations was assayed weekly, and the results are summarized in Table 1. The values obtained demonstrated the excellent reproducibility of this assay system. Variations in the ND_{50} value did not exceed about 10% of the mean and the standard deviation for serum 2 was as low as 5.1%.

Effect of the dose of challenge virus. A report of a study on the vaccinia virus plaque reduction method, on HeLa cell monolayers, showed that a reduction in the dose of challenge virus to 1/10 resulted in an almost 3-fold increase of the ND_{50} titre obtained (Kitamura et al., 1964). In the present studies, this effect of the challenge virus dose was checked by assaying a serum from a recently revaccinated male adult against different doses of the challenge virus. As summarized in Table 2, there was no significant rise in the ND_{50} titre in response to the reduced dose of challenge virus. This was confirmed by the routine assay practices in the later studies, in which the standard IRS assayed as the positive control at every assay showed practically identical values against doses of challenge virus that varied significantly. The discrepancy between the present results and those of the former studies will be discussed later.

Comparisons with other assay systems. To compare the different assay procedures available, three antibody preparations were submitted to three different variants of the neutralization test that had been reported to be satisfactory, i.e., focus reduction of VHE variola virus, pock reduction of VHE, and plaque reduction of DIE vaccinia virus (Kitamura et al., 1964). For the first two procedures, the reaction mixtures of diluted antibody and VHE virus were inoculated on to HeLa cell monolayers and dropped CAMs. Two plates or 6 CAMs were used for each reaction mixture. As shown in Fig. 2, the three antibody preparations gave similar results in the different assay systems. The variola focus reduction and vaccinia plaque reduction assays resulted in practically parallel sigmoid curves, the steep portions

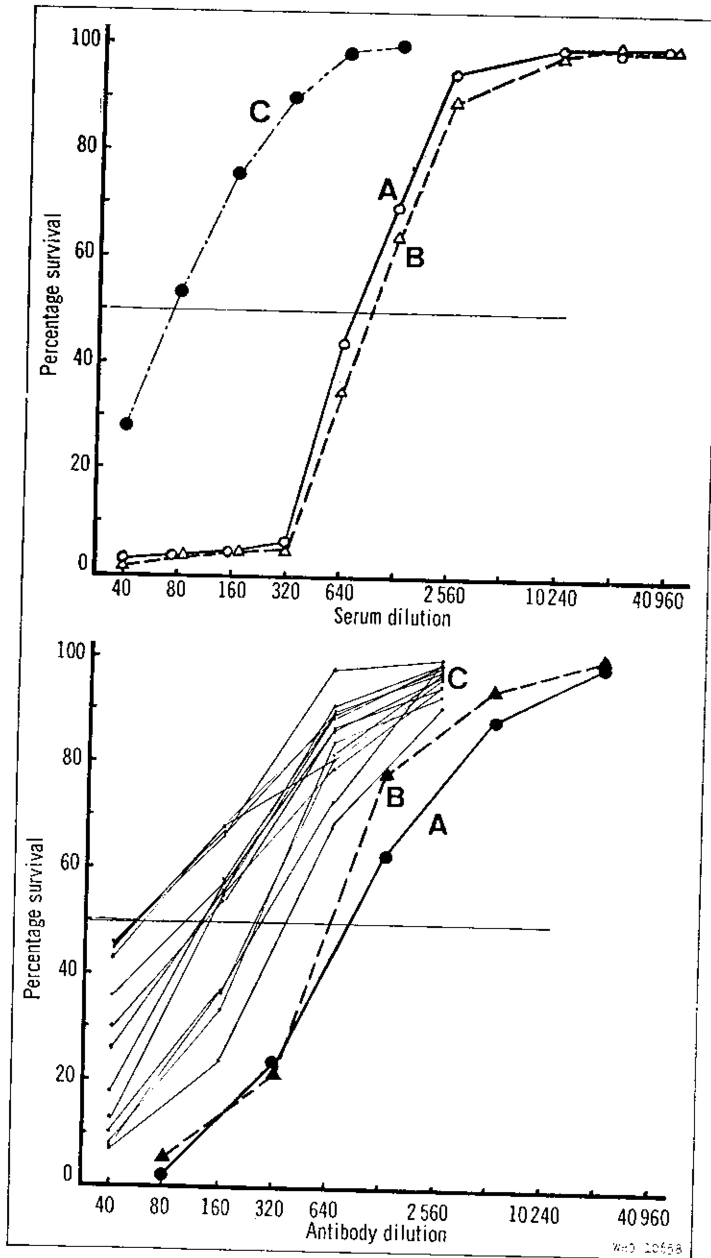

Fig. 1. The neutralization curves resulting from tests with logarithmic dilutions of the antibody preparations. *Top*—two-fold dilutions of 3 representative sera (100 % survival value: 324 foci/plate): A, antivaccinia rabbit serum; B, antivariola rabbit serum; C, serum from a human female adult, 12 years after the last smallpox vaccination. *Bottom*—four-fold dilutions of 3 groups of human sera or VIG from them (100 % survival value: 205 foci/plate): A, VIG preparation from a pool of sera from recently vaccinated adults; B, International Standard for Anti-Smallpox Serum; C, 13 pools of sera from adults revaccinated within the previous 6 months.

Table 1. ND_{50} titres obtained for two preparations of human serum assayed five times at weekly intervals

Assay no.	Dose of challenge virus [a]	Serum 1 [b]	Serum 2 [b]
1	410	505	76
2	390	—	77
3	411	560	69
4	489	—	81
5	436	450	75
average	—	505	76 $\sigma = 3.9$ (5.1 %)

[a] Number of foci on control plates inoculated with the NRS-virus mixture.
[b] Serum 1 = the International Standard for Anti-Smallpox Serum. Serum 2 = serum from one of the authors (T.K.), repeatedly vaccinated.

Table 2. Relation between the challenge virus dose and the ND_{50} titre obtained

Challenge virus dose [a]	ND_{50} titre obtained
764	88
578	84
422	94
181	86
61	105
30	99

[a] Number of foci in the control plates of NRS-virus mixture.

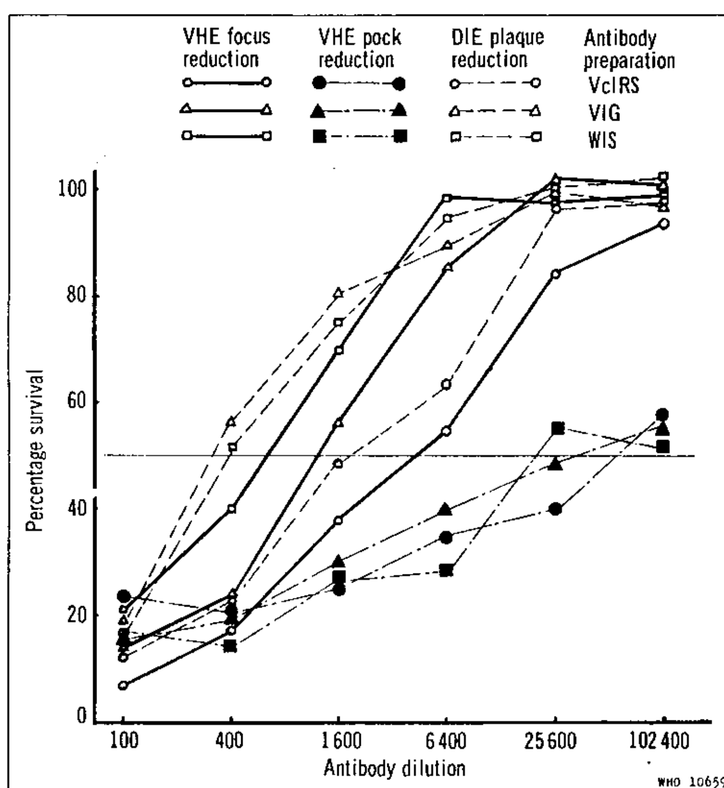

Fig. 2. Comparative assays of neutralizing antibody by the focus reduction and pock reduction methods with variola virus and the plaque reduction method with vaccinia virus. The 100 % survival values were 304 foci/plate, 202 pocks/CAM and 134 plaques/plate respectively.

of which had identical gradients; the results of the latter assay showed a shift to the left, however, to the lower end of the dilution scale. There was no substantial difference between the curves of antivaccinia (VcIRS and VIG) and antivariola (WIS) antibodies against the two viruses. The curves for the variola pock reduction assay, however, were significantly different from those of the *in vitro* assays; they revealed relatively incomplete neutralization in the lower dilution range, and the gradient of the linear portion of the curve was not so steep, resulting in higher ND_{50} titres with somewhat larger fluctuations. A pock reduction assay was also attempted with antibody–DIE mixtures in the present series of experiments, but it was practically impossible to draw a sigmoid curve because of the limited number of pocks per CAM countable and the resulting larger standard error (about 30% of the mean). Thus it may be concluded that the values obtained using the variola focus reduction method are identical with those obtained using the vaccinia plaque reduction method. The reproducibility of the two methods is

Table 3. Local skin reaction and antibody rise after revaccination in relation to vaccine potency

Vaccine potency (pock-forming units/ml)	Reaction subgroup [a]	No. of cases classified	Take rate [b] (%)	Average ND_{50} titre		B/A ($\log_{10}$)
				Pre-vaccination (A)	Post-vaccination (B)	
1.30×10^8 (10^0)	+	13		16.9	74.5	4.31 (0.64)
	±	9		17.3	59.8	3.44 (0.54)
	−	3		5.3	19.7	3.72 (0.57)
subtotal		25	88.0	15.6 [c]	62.7	4.02 (0.60)
1.30×10^7 (10^1)	+	8		19.6	76.1	3.89 (0.59)
	±	6		22.7	41.3	1.82 (0.26)
	−	17		9.8	39.1	3.99 (0.60)
subtotal		31	45.1	14.5 [c]	49.4	3.41 (0.53)
1.30×10^6 (10^2)	+	3		5.3	43.3	8.17 (0.91)
	±	3		19.3	39.3	2.04 (0.31)
	−	24		17.1	45.7	2.67 (0.43)
subtotal		30	20.0	16.2 [c]	42.1	2.59 (0.41)
1.30×10^5 (10^3)	+	0		—	—	—
	±	1		2.0	2.0	1.00 (0.00)
	−	13		14.7	35.5	2.41 (0.38)
subtotal		14	7.1	13.8 [c]	33.2	2.40 (0.38)
arithmetic mean of all cases studied				15.2		

[a] The average size of the reddening, for each vaccine group, in cases classified as " −reaction " was as follows:

10^0 . . . . 6.7 × 6.0 mm
10^1 . . . . 3.7 × 3.8 mm
10^2 . . . . 3.3 × 3.3 mm
10^3 . . . . 0.6 × 0.5 mm

[b] Reactions + and ± were taken together as " major reactions " and were regarded as a successful take.

[c] Arithmetic mean of the ND_{50} titres of all the members of the group.

identical but the former method is more sensitive and gives higher ND_{50} titres; both methods are much superior to the pock reduction method.

Neutralizing antibody response to smallpox revaccination by the focus reduction method

Groups of about 30 adult females were inoculated with 10-fold serial dilutions of a calf-lymph type smallpox vaccine and were examined for local skin reactions 7 days after inoculation. Neutralizing antibody levels were determined before, and 6 weeks after, the vaccination as described earlier.

General aspects of the response. The correlation between the potency of the vaccine (pock-forming units/ml) and the percentage of successful takes (Table 3) followed the same type of sigmoid curve as has been obtained at the primary vaccination of babies (Kitamura et al., 1963) or young male adults (Cockburn et al., 1957) but the level of potency necessary to give a successful take at the revaccination seemed to be much higher than at the primary vaccination. The take rate was as low as 20.0% with a vaccine of 1.3×10^6 pock-forming units/ml potency, which would give a take rate higher than 90% at primary vaccination. Even with a vaccine having a potency as high as 1.3×10^8 pock-forming units/ml, the take rate was still only 88%; this suggests that to achieve a successful take in more than 90% of a revaccinated population a vaccine should have a potency higher than 1.3×10^8 pock-forming units/ml. Factors affecting the take rate in a revaccinated population will be discussed in later sections of this paper. This trial also showed that the average neutralizing antibody level in a group of adults 8 years after their last vaccination (the third vaccination under the present national regulations in Japan) was 15.2 ND_{50}, a figure that might be of value for the purpose of surveying the immunity status of a community, if the survey were performed on a random sample of the population.

Potency of the vaccine and the antibody response. The correlation between the ND_{50} titres before and after vaccination in each of the experiments is shown in Fig. 3. A significant rise was demonstrated in all

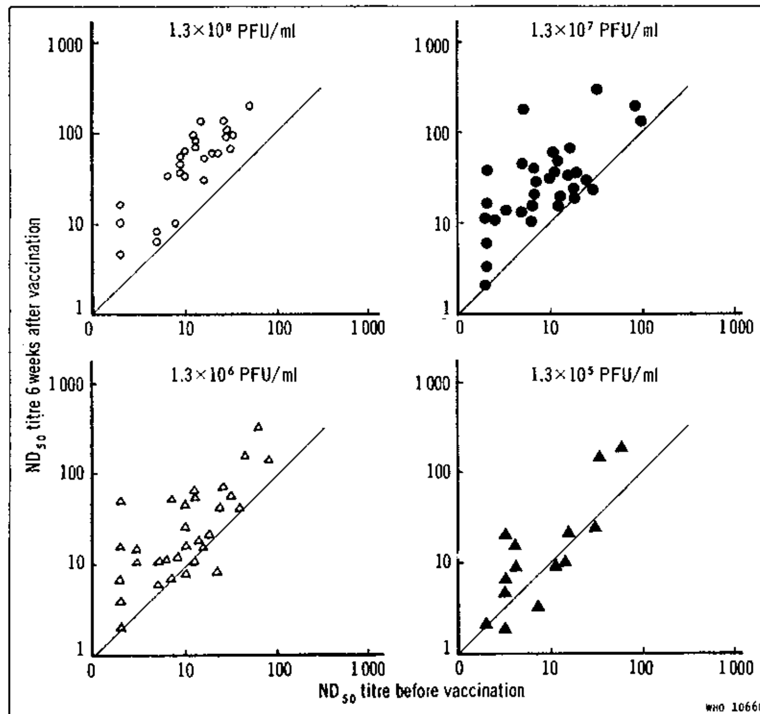

Fig. 3. Relation between ND_{50} titres before and after the vaccination with vaccines of different potencies.

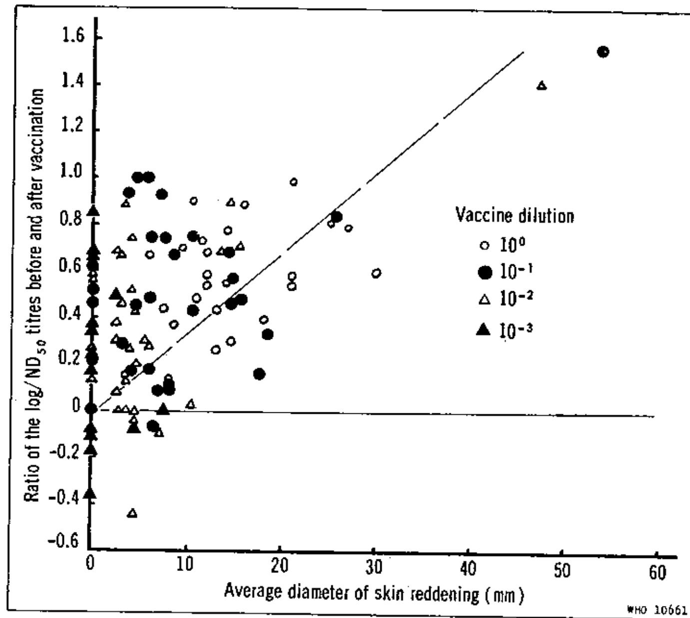

Fig. 4. Relation between the increase in neutralizing antibody titre and the diameter of the skin reddening after vaccination with different vaccine dilutions.

persons inoculated with undiluted vaccine (10^0), but the number of cases without such a rise increased with reduction of the potency. This is demonstrated in Table 3 by comparing the average ND_{50} titres of each vaccine group. Starting from a practically identical level of titre before vaccination, the titres increased in the 6 weeks after vaccination, in the ratios of 4.02 (10^0), 3.41 (10^{-1}), 2.59 (10^{-2}), and 2.40 (10^{-3}). It should be noted here that vaccination with the 10^{-3} dilution (1.3×10^6 pock-forming units/ml), which resulted in no successful takes in terms of skin reaction, gave significant rises in antibody titre by as much as 2.40 times the initial value. This could not have been accounted for by an identical level of significant local reaction being classified as equivocal in some potency groups, since the average diameter of the local reddening in the cases with reactions classified as " – reactions " (see footnote to Table 3) clearly demonstrated that the 10^{-3}-vaccine group had the smallest area of reddening. This finding will be discussed further in the following section of this paper.

Local skin reaction and antibody response. An earlier study with undiluted vaccine using the vaccinia plaque reduction test as the antibody assay procedure (Kitamura et al., 1964) suggested a direct correlation between the increase of the log ND_{50} titre and the diameter of the skin reaction. In Fig. 4 the relation between the diameter of skin reddening and the increase in the neutralizing antibody titre in the present study has been plotted for all vaccine dilutions. This figure shows that the vaccines with potencies higher than 10^8 pock-forming units/ml (10^0) gave a distribution with a correlation coefficient of 0.45, but that the correlation coefficient was lower with vaccines of lower potency; with these vaccines significant rises in antibody titre were accompanied by weak or negligible skin reactions. Table 4 shows the average increase in antibody titre for all the subjects

Table 4. Relation between the local skin reaction and the rise in antibody level

Reaction group	No. of cases classified	Average ND_{50} titre		B/A ($\log_{10}$)
		Pre-vaccination (A)	Post-vaccination (B)	
+	24	16.4	71.2	4.34 (0.64)
±	19	18.5	53.8	2.91 (0.46)
–	57	13.8	40.1	2.90 (0.46)

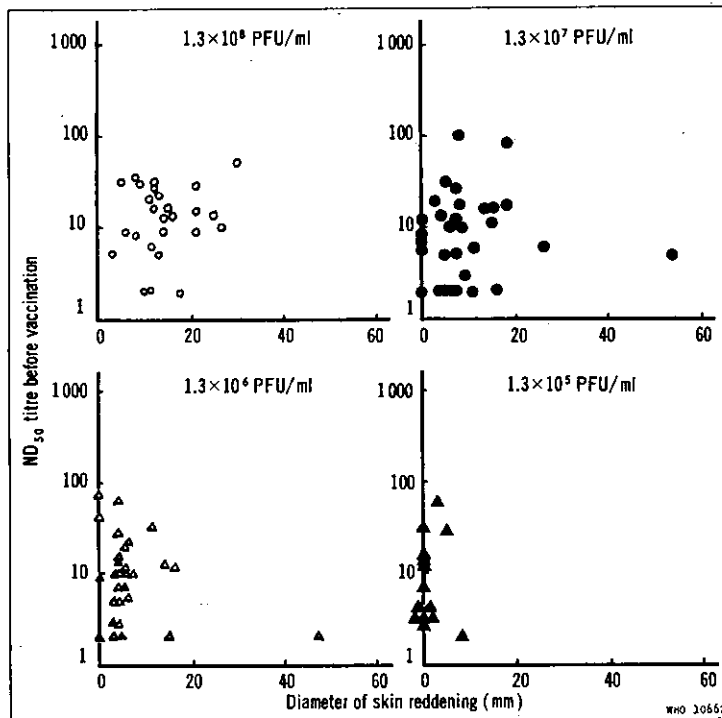

Fig. 5. Relation between skin reaction and neutralizing antibody titre before vaccination with vaccines of different potencies.

grouped according to their reaction category. The 57 cases with a " – reaction " showed an almost 3-fold increase in titre, identical with that of ± group. It may be concluded that in a subject having some residual immunity, revaccination will strengthen the immunity irrespective of the local skin reaction after the revaccination.

Skin reaction and the initial level of antibody. It has previously been thought that the absence of a reaction at revaccination meant that the individual had sufficient immunity against poxvirus infection and that he could be classified as well protected against smallpox. This conclusion was brought into question, however, by the results of earlier studies that showed that there were no significant differences in neutralizing antibody levels at the time of revaccination between groups with good takes and those with no response (Kitamura et al., 1964). In the present study, there was no significant correlation between the skin-reaction grouping and the antibody titre before revaccination, as shown in Table 3. The data for each individual are plotted in Fig. 5. The groups vaccinated with vaccine dilutions 10^0 and 10^{-1} (potency higher than 1.3×10^7 pock-forming units/ml) both showed an identical, random distribution, suggesting that the skin reaction to revaccination with a vaccine of a potency higher than a certain level is determined by some factor other than the level of circulating antibody. Reactions to the weaker vaccines (dilutions 10^{-2} and 10^{-3}), however, did seem to be dependent to some extent on the neutralizing antibody level, the points being distributed approximately along the hyperbola that would result from a reciprocal relationship. These results could be interpreted as implying that a strong skin reaction after revaccination with a vaccine of low potency (of the order of 10^6 pock-forming units/ml or less) indicates weak initial immunity.

DISCUSSION

A new procedure for assaying the neutralizing antibody against variola virus *in vitro* has been developed in the present study employing a macroscopic focus counting method on HeLa cell cultures in place of

plaque counting of vaccinia virus (Kitamura et al., 1964). In several neutralization test systems with animal viruses it has been recommended that complement should be added. In the present study, it was found that the addition of complement in high concentration could increase the ND_{50} titre in serum preparations after the primary vaccination. However, the inhibitor in the fresh guinea-pig serum used as the source of complement was highly damaging to the variola virus at a complement dose level that was effective in the neutralization test: this resulted in a reduction of the titre of the challenge virus by more than 60–70% (Kitamura, T. & Miyagawa, Y., unpublished data), as suggested by McCarthy & Germer (1952) for normal human serum. Thus it was decided that the addition of complement to the reaction mixture should be avoided in our study until a preparation or source of complement free of inhibitor against variola virus could be developed. The absence of a significant effect of the dose of challenge virus on the ND_{60} titre (Table 2) is difficult to explain at present. The ratio between the number of focus-forming units and the number of physical particles was supposed to be equivalent to the ratio involving the number of plaque-forming units, as it had been observed that the ratio of plaque-forming units to focus-forming units for variola virus was identical with the ratio of pock-forming units to plaque-forming units for vaccinia virus (Kitamura, 1968). The vaccinia plaque reduction method revealed a clear relationship between the dose of challenge virus and the resulting ND_{60} titre of a single antibody preparation—a 90% reduction in the former giving a 3-fold increase in the latter value. Details of the interactions between the virus and the antibody molecule will have to be elucidated before any interpretation can be made.

The high degree of reproducibility of the ND_{50} values obtained in the present study (Table 1) indicates the possibility of determining the number of international units (IU) in an antipox neutralizing antibody preparation directly from the ND_{50} value. In the present study, the International Standard for Anti-Smallpox Serum, designated arbitrarily to contain 1 000 IU/ml (Anderson & Skegg, 1970) gave an average titre of 505 ND_{50} and thus it would be possible to convert this value into IU by the equation: 1 ND_{50} = 1.98 IU, if the present observations are confirmed by further independent tests by other workers. The poor results of the pock reduction method with both vaccinia and variola viruses were confirmed in the present work. There was no improvement of the situation by increasing the number of CAMs per reaction mixture, as this resulted in a flatter rise of the straight portion of the sigmoid curve and in fluctuation of the neutralization rates, to give fluctuations of the ND_{50} values in individual assays 2 or 3 times greater or smaller than the mean. This may indicate a substantial difference between the *in ovo* and *in vitro* systems, which may be caused by the fragile nature of the viral entities that form the pocks on the CAMs: this explanation is implied by the appearance of the early-focus-former (EFF) prior to the appearance of the pock former in the growth curve of VHE virus in HeLa cells (Kitamura & Yotsuyanagi, 1968). The EFF was more labile to protease digestion but was more stable to heat inactivation and neutralizing antibody than the pock former. The number of pock-forming units might have been reduced and, even with the fluctuating levels of the neutralization, this would have resulted in apparently higher but less reproducible ND_{50} titres.

In view of the excellent reproducibility of the present assay method, it might be possible, during the surveillance of several communities in a smallpox endemic area over a period of several years, by the assay of neutralizing antibody in persons associated with smallpox cases, to correlate the antibody level with the occurrence of disease, and thus to investigate statistically whether a particular level of neutralizing antibody is sufficient to prevent the disease. This study has confirmed that, in normal individuals, a strong skin reaction after revaccination ensures a greater rise in the neutralizing antibody titre. Another remarkable feature in the present study, however, was the fact that a very weak or negligible skin reaction could also be associated with a significant rise in antibody. This observation may be encouraging for public health authorities in that it promises an effective strengthening of the immunity in individuals who have been vaccinated once before, by a single revaccination regardless of the skin reaction at that time.

Such a generalization from the present results with the Ikeda strain as the vaccine virus, which has recently been reported to be more reactogenic than other popular strains—e.g., Lister (Elstree), EM-63 (Ecuador), and NY (New York Board of Health)—may be justified in view of the results of a recent study (Takatsu, unpublished data). In that study, 3 groups of about 2 000 babies, ranging from 2 to 24 months of age, were given a primary vaccination with vaccines made from Ikeda, Lister, or EM-63 strain vaccinia

viruses, adjusted to identical potency (1.0×10^8 pock-forming units/ml), by the multiple pressure method with bifurcated needles. The inoculations and observations were carried out by more than 50 leading paediatricians and virologists with rigid standardization of the criteria and techniques. There were differences between these virus strains in the characteristics of the local skin reaction: the Ikeda strain produced most induration and double reddening, the Lister strain the least, and the EM-63 strain was intermediate; but the proportions of major reactions (successful takes), the occurrence of pathological exanthema and systemic fever, the duration of the fever, and the HI antibody response revealed no differences between 3 strains. The neutralizing antibody assay carried out on a small number of sera from the above study suggested an identical relationship between the skin reaction and the final antibody level for the 3 strains of the vaccine virus.

The results from the groups vaccinated with vaccines of lower potency (10^{-2} and 10^{-3} dilutions) suggested that a successful take after vaccination with a vaccine having a potency of 10^6 pock-forming units/ml or less may imply a low level of immunity at the time of revaccination (Fig. 5). The failure to take, however, may be a result of the complex conditions that exist at the inoculation locus, including the cellular immunity, the circulating antibody level, or the heat-labile virus inhibitor suggested by McCarthy & Germer (1952). At least, this fact may be useful in detecting the individual with lower immunity by revaccinating with a vaccine of critical potency, supposedly around 10^6 pock-forming units/ml. The levels of complement-fixing and haemagglutination-inhibiting antibodies were also assayed in the sera of the present vaccination study. There was almost no case that showed a detectable level of both antibodies at the dilution of 1 : 5 and no case was observed that had experienced a significant rise in the levels of both antibodies as a result of revaccination. This observation appears to be in agreement with that of McCarthy et al. (1958b) that HI and CF antibodies did not usually increase or develop after revaccination.

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RÉSUMÉ

TITRAGE DES ANTICORPS NEUTRALISANTS ANTIVARIOLIQUES PAR LA MESURE DE LA RÉDUCTION DU NOMBRE DES FOYERS D'HYPERPLASIE SUR CULTURES DE CELLULES HELA; APPLICATION À L'ÉTUDE DE LA RÉPONSE IMMUNITAIRE APRÈS REVACCINATION PAR DES VACCINS ANTIVARIOLIQUES D'ACTIVITÉ VARIABLE

Les auteurs décrivent une méthode de titrage des anticorps neutralisants antivarioliques basée sur la mesure de la réduction du nombre des foyers d'hyperplasie dans une culture de cellules HeLa en couche monocellulaire. La corrélation entre le taux de dilution d'une préparation d'anticorps et le taux de survie du virus d'épreuve affecte la forme d'une courbe sigmoïde typique. On peut de la sorte déterminer avec une exactitude et une reproductibilité très satisfaisantes la dose d'anticorps provoquant une réduction de 50% du nombre des foyers (DN_{50}).

La DN_{50} d'une préparation donnée d'anticorps ne subit pas de modification significative lorsqu'on utilise des doses de virus d'épreuve variant de 30 à 764 PFU (unités formatrices de plages) par plaque. La mesure de la réduction du nombre des foyers permet d'obtenir régulièrement des valeurs de la DN_{50} plus élevées que celles fournies par la méthode standard de neutralisation du virus vaccinal. Quant au titrage basé sur la réduction du nombre des plages du virus variolique, il donne des valeurs de la DN_{50} plus élevées, mais il se montre moins fiable et moins reproductible.

La méthode a servi à étudier la réponse immunitaire chez des femmes revaccinées contre la variole 8 ans au moins après la dernière inoculation. Réparties en groupes de 30 environ, elles ont reçu une dose de vaccin antivariolique non dilué ($1,3 \times 10^8$ PFU/ml), ou dilué au 1/10, au 1/100 ou au 1/1000. On a recherché les corrélations entre les titres d'anticorps mesurés avant et 6 semaines après la vaccination et l'intensité des réactions cutanées locales au 7e jour.

Les taux de prise ont été respectivement de 88,0 % (vaccin non dilué), 45,1 % (vaccin au 1/10), 20,0 %

(vaccin au 1/100) et 7,1 % (vaccin au 1/1000). Une hausse très appréciable des titres d'anticorps est survenue après administration de vaccin non dilué, mais les autres préparations, dont le vaccin dilué au 1/1000, ont elles aussi provoqué une réponse immunitaire notable. La corrélation entre le diamètre de la réaction cutanée et la hausse des titres s'est révélée satisfaisante après inoculation de vaccin non dilué, mais faible après emploi des vaccins moins actifs. Chez des sujets présentant une réaction locale nulle ou très modérée, on a cependant constaté une hausse notable des titres, ce qui donne à croire qu'un certain degré d'immunité résiduelle peut être renforcé par la vaccination, quelle que soit l'intensité des signes cutanés. Il n'existait aucun lien entre le niveau de l'immunité prévaccinale et le succès ou l'échec de la revaccination pratiquée à l'aide des vaccins très ou moyennement actifs. En revanche, on a relevé une relation directe entre l'intensité de la réaction locale et les titres prévaccinaux chez les sujets recevant la préparation du faible pouvoir antigénique.

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