

Relationship between Pock Counts on Chorio-allantoic Membrane and Percentages of "Takes" in Primary Vaccination of Human Beings with Two Smallpox Vaccines

M. F. POLAK,¹ L. M. BRANS,² B. J. W. BEUNDERS³
& A. R. VAN DER WERFF⁴

In view of doubts as to the potency level required to obtain nearly 100% successful vaccinations with the glycerolated smallpox vaccine prepared by the Netherlands National Institute of Public Health, its dose-effect relationship was compared with that of a glycerolated lymph from the Lister Institute of Preventive Medicine, England. The potency was estimated from pock counts on the chorio-allantoic membrane of chick embryos and the effect was judged from the "take" rate obtained in the primary vaccination of soldiers, the data being subjected to probit analysis.

The relationship observed between the number of pock-forming units and the percentage of successful primary vaccinations was nearly identical with the two vaccines. The 50% effective doses were found to be $1.3-1.4 \times 10^6$ PFU/ml and the 99% doses to be $4.1-4.3 \times 10^7$ PFU/ml.

A WHO Study Group on Requirements for Smallpox Vaccine (1959) has described three methods to test the potency of vaccine in the laboratory. In one of these, the numbers of pocks developing on the chorio-allantoic membranes of chick embryos after application of suitable vaccine dilutions and incubation are counted. The number of pock-forming units in 1 ml of undiluted vaccine (PFU/ml) is computed from these data and should exceed 5×10^7 .

Cockburn et al. (1957) assessed the relation between the potency as determined by pock counts on the chorio-allantoic membranes and the percentage of "takes" observed after primary vaccination of young adults. Their vaccine Q was a dried calf-lymph preparation stored at 37°C in different sublots for 4, 8, 16 and 32 weeks. The results were analysed by the probit method. At a vaccine po-

tency of 1.3×10^7 PFU/ml 99% "takes" were achieved, the 50% point being determined at 3.0×10^5 P.F.U./ml. The authors conclude that, allowing for possible errors in their experiments, vaccines which give a pock count of 10^8 infective units per ml will give the highest possible rate of successful primary vaccinations.

The results obtained in a study of primary vaccination of infants performed in the Netherlands (Polak & Brans, 1962) were not very satisfactory in that there was a certain frequency of failures with glycerolated vaccine of 2×10^8 PFU/ml. The success rate in infants of 6-11 months of age amounted to 90%-95%, excluding the data from one vaccinator group with clearly lower results. Although other causes might have been responsible, it was not justifiable to exclude a possible difference in infectivity between our vaccine strain and the strain used in production of vaccine Q, referred to above. It was therefore decided to determine the dose-effect relationship for the vaccine in common use in the Netherlands, prepared in the National Institute of Public Health at Utrecht, simultaneously with a glycerolated lymph produced by the Lister Institute of Preventive Medicine in England.

¹ Epidemiological Service, National Institute of Public Health, Utrecht, Netherlands.

² Smallpox Vaccine Laboratory, National Institute of Public Health, Utrecht, Netherlands.

³ Colonel, Department of Preventive Medicine and Hygiene, Medical Corps, Royal Netherlands Army.

⁴ Captain, Medical Corps, Royal Netherlands Army.

MATERIALS AND METHODS

Vaccines

Dr C. Kaplan kindly supplied a batch (No. 3768) of glycerolated sheep lymph, prepared at the Lister Institute of Preventive Medicine. This product is designated vaccine L.

A batch (No. 59134) was chosen from the routine production of glycerolated calf lymph prepared in the National Institute of Public Health in the Netherlands. This product (vaccine R) has been obtained through one rabbit and three calf passages from vaccinia virus No. 9-521, dated 21.9.54 and supplied in 1955 by Dr J. Ørskov, Statens Serum-institut, Copenhagen.

The two vaccine lots were distributed into a number of separate tubes and stored at -70°C so as to avoid deterioration of the product as much as possible. In the course of the trial the contents of a tube were thawed according to the needs for potency tests, vaccinations, or both.

Potency tests

Ten chick embryos of 13 days' incubation were used per test. The dropped chorio-allantoic membrane was inoculated with 0.1 ml of an appropriate vaccine dilution and after two days' further incubation the number of pocks per membrane was counted. The number of PFU/ml was computed from the mean count per series of membranes—after discarding damaged membranes and dead embryos—and the dilution applied.

Both vaccines were tested in duplicate on the same day. The duplicates were based on separate dilution series. By repeating these series of four tests on a number of days before and during the vaccination trials in humans, it was possible to gauge the significance of inter-dilution and inter-day variations. McIlvaine buffer (0.004 M phosphate, pH 7.2) was used as diluting agent.

Vaccinations

In order to obtain graded doses of vaccine to be applied in the vaccination trial, vaccines L and R were diluted with 40% glycerol in McIlvaine buffer. Dilutions were made in the morning and the respective vaccine bottles were identified by code number. The bottles were transported on ice to the vaccination centre, where vaccinations were performed in the afternoon. The vaccine dilutions were kept on ice as long as was convenient during each vaccination session. The vaccinations were carried

out with great care by two experienced inoculators. Two linear scratches of about 3 mm were made on the left upper arm and the vaccine was rubbed into the lesions. About one minute later, a piece of gauze was applied and the subject received, by intramuscular injection in the opposite arm, 2 ml of 16% vaccinia hyperimmune gamma-globulin for the prevention of post-vaccinal encephalitis.¹

The trial was carried out from October 1960 until January 1961 in an army unit among three groups of about 120 young soldiers, who, from their medical histories and from inspection of the skin at the customary inoculation sites, appeared not to have been vaccinated previously.

For each session a vaccination programme was drawn up in advance in order to ensure the greatest possible comparability between vaccinators and between corresponding vaccine dilutions. Each vaccinator did five successive inoculations with the lymph from one bottle. In the design of the experiment some preference was given to the vaccine dilutions near the expected 50% effective dose, as may be seen from the results in Table 3 below.

The men were inoculated in succession as they presented themselves for vaccination. We assume that no bias entered into these procedures, although the vaccinators could, of course, distinguish between lymphs of high and low dilutions.

The results were recorded on the seventh day after vaccination as positive (1 or 2 vesicles) or negative (no vesicles). The readers had no information about the vaccine or dilution used.

RESULTS OF POTENCY TESTS ON CHICK EMBRYOS

Duplicate tests for each vaccine were carried out on 39 days, so that 78 potency values are available per vaccine. Geometric mean values were calculated for a preliminary series of 40 tests and three series of, respectively, 16, 12 and 10 tests were performed concurrently with three vaccination experiments. As shown in Table 1, the range of potency ratios of vaccine L to vaccine R varied from 2.6:1 to 3.1:1. The average ratio for 78 tests per vaccine came to 2.7:1 and the non-weighted average for the last three series amounted to 2.9:1.

It was intended to perform the tests at a level of 10-15 pocks per membrane. The early choice of dilution led, however, in the long run to average counts of slightly under 10 with both vaccines but

¹ See the article by W. Nanning on page 317 of this issue.

TABLE 1
POTENCY TESTS OF VACCINES L AND R ON CHORIO-ALLANTOIC MEMBRANE

Test series	Tests per vaccine	Geometric mean potency ($\times 10^4$ PFU/ml)		Potency ratio L : R
		Vaccine L	Vaccine R	
1. Preliminary series	40	9.87	3.85	2.6:1
2. Concurrent with vaccination experiment 1	16	9.91	3.20	3.1:1
3. Concurrent with vaccination experiment 2	12	9.69	3.31	2.9:1
4. Concurrent with vaccination experiment 3	10	8.92	3.40	2.6:1
Weighted average of series 1, 2, 3 and 4	78	9.72	3.56	2.7:1
Unweighted average of series 2, 3 and 4	38	9.50	3.30	2.9:1

the serial dilution end-points were nevertheless not changed.

The pock counts of the first series of 40 tests per vaccine were analysed for the degree of fitting to a theoretically expected Poisson distribution.

Although general experience had led us to anticipate that there would be some divergence from this distribution, we were interested to see whether there was any difference in this respect between vaccines L and R. The results of analysis of variance

TABLE 2
VARIANCE COMPONENTS FROM A SERIES OF 40 CAM TITRATIONS PER VACCINE^a

Source of variation	Variance components		Standard deviation (= square root of variance component)	
	Vaccine L	Vaccine R	Vaccine L	Vaccine R
Poisson	9.25	9.93	3.04	3.15
Between eggs	5.24	5.98	2.29	2.45
Between dilution series	1.83	1.14	1.35	1.07
Between days	3.19	3.24	1.79	1.80

^a Two tests on 10 eggs each were carried out daily for 20 days with each vaccine.

on 388 egg counts per vaccine (12 eggs had to be discarded for such reasons as early embryo death or membrane damage) are given in Table 2. As may be seen, sources of extra variation are active between eggs within a test series, as well as between dilution series on the same day and between days. All these sources are to be considered significant ($P=0.05$ or less). It seems, however, that for both vaccines these sources are of about equal importance.

RESULTS OF PRIMARY VACCINATION OF HUMAN BEINGS

On account of the results of the preliminary potency tests (Table 1) and the outcome of a pilot trial with 100 men, the following dilutions were chosen for the three vaccination groups of the main trial:

Vaccine L	Vaccine R
1: 100	1: 40
1: 500	1: 200
1: 2 500	1: 1 000
1: 12 500	1: 5 000

The success rates are given in Table 3. As the agreement between vaccinators regarding "take" rates was good, these are not shown separately for each vaccinator.

Notwithstanding the efforts to select men for primary vaccination only, accelerated reactions recorded as "positive" were observed in three

TABLE 3
RESULTS^a OF PRIMARY VACCINATION (TWO SCRATCHES) WITH DILUTED VACCINES

Dilution	Trial group			Total	
	1	2	3	No.	%
Vaccine L					
1: 100	11/12	10/10	12/16	33/38	87
1: 500	15/20	11/20	10/20	36/60	60
1: 2 500	5/20	5/19	3/20	13/59	22
1: 12 500	0/10	0/10	ND	0/20	0
Vaccine R					
1: 40	10/10	9/10	14/15	33/35	94
1: 200	10/20	13/20	9/18	32/58	55
1: 1 000	7/20	3/20	1/20	11/60	18
1: 5 000	0/10	0/10	ND	0/20	0

^a Expressed as the number of "takes" (at least one pock) over the number of persons vaccinated. ND = not done.

TABLE 4
RESULTS OF PROBIT ANALYSIS

Group	Vaccine L			Vaccine R			Difference of slope (P)
	b	ED ₅₀ ^a	χ ²	b	ED ₅₀ ^a	χ ²	
1	-1.59	3.01	3.93	-1.25	2.52	2.01	0.5
2	-1.96	2.89	1.09	-1.82	2.45	0.49	0.7
3	-1.22	2.62	0.16	-2.16	2.28	0.02	0.03
1, 2 and 3	-1.45	2.85	0.50	-1.72	2.42	1.14	0.2
1, 2 and 3 with common b values for L and R	-1.55	2.85		-1.55	2.42		

^a ED₅₀ expressed as log dilution.

cases (with vaccine L at dilutions 1:500 and 1:2500 and with vaccine R at dilution 1:40). The selection procedure had thus not been completely successful. This might have affected some numerator values in Table 3 in that some men responded negatively not on account of their innate tolerance but because of acquired specific immunity. The significance of this factor may be illustrated by subsequent experience of the same team in the course of alternately administering several full potency vaccines. The same criteria as before were used to select candidates for primary vaccination. Indeed, if there was any difference, it was that in the later trial these criteria were less rigorously applied. Among a total of 806 "primary" vaccinations, all read as positive, accelerated reactions or reactions of immunity were observed in 19 persons and regular primary vaccinia reactions in 787. The same rate applied to the 158 successful vaccinations in the present trial would give three or four non-primary reactions—a figure which agrees well with the three accelerated reactions actually recorded.

Table 4 summarizes the results of probit analysis on the data of Table 3. The low χ² values give evidence of a good fit between the observed points and the regression lines. For groups 1 and 2 the slopes for both vaccines are in good agreement, but in the third trial group the b values differ significantly (P=0.03). It is not unreasonable to consider this as chance variation, particularly as analysis of covariance, testing slope differences, gave high P values (0.5, 0.1, 0.3 and 0.2, respectively) in the following instances:

- probit regression lines of 3 groups with vaccine L,
- probit regression lines of 3 groups with vaccine R,
- six regression lines mentioned under (a) and (b), and
- 3 groups with vaccine L against 3 groups with vaccine R.

Estimation of a common slope for both vaccines gives a b value of -1.55 (SD=0.15). For vaccine L, the ED₅₀, expressed as log dilution, amounts then to 2.85, and for vaccine R to 2.42.

The observed points and probit regression lines are shown in Fig. 1. The following formulae hold for regression lines with a common slope:

Vaccine L: probit = 9.402 - 1.546 log dilution.

Vaccine R: probit = 8.727 - 1.546 log dilution.

RELATION BETWEEN POTENCY ON CHICK EMBRYOS AND "TAKE" RATE IN HUMAN BEINGS

For vaccine L, as shown in the last row of Table 4, the 50% effective dose, expressed as log dilution, is 0.43 higher than for vaccine R. The 95% confidence limits are at 0.214 and 0.626. This means a 50% effective dose ratio for L:R of 2.69:1 with 95% confidence limits at 1.64:1 and 4.23:1. Potency ratios assessed on chick embryos (Table 1) are

FIG. 1
PROBIT-LOG-DILUTION REGRESSION LINES AND OBSERVED POINTS

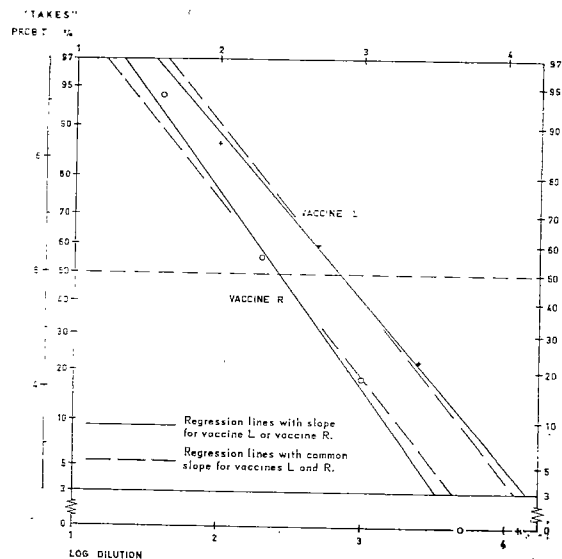


TABLE 5
THEORETICAL RELATION BETWEEN PERCENTAGE
"TAKES" IN HUMANS AND POTENCY ON CHORIO-
ALLANTOIC MEMBRANE

Percentage "takes"	Potency on CAM	
	Vaccine L	Vaccine R
99	4.3×10^7	4.1×10^7
90	9.1×10^6	8.6×10^6
50	1.4×10^6	1.3×10^6
10	2.0×10^5	1.9×10^5
1	4.2×10^4	4.0×10^4

clearly within these limits.¹ We may conclude, therefore, that dose-effect relationships as measured in this trial do not differ significantly for vaccines L and R.

To generalize the relationship between potency and effect, log dilution values must be transformed to pock-forming units per ml. We take as potencies for non-diluted lymphs the data from the last line of Table 1:

Potency, vaccine L: 9.50×10^8 PFU/ml.

Potency, vaccine R: 3.30×10^8 PFU/ml.

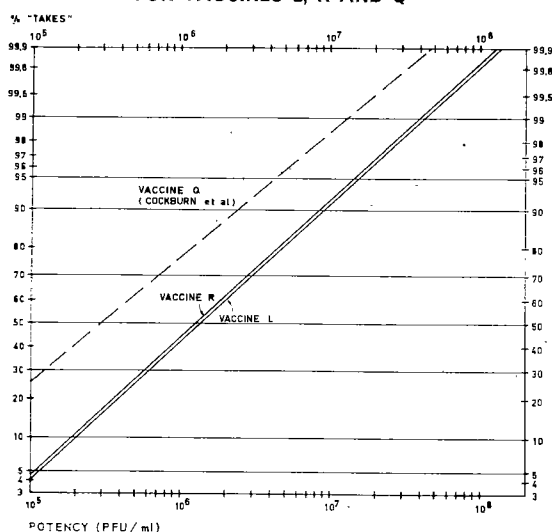
The best estimates of the relation between percentage "takes" in human beings and potency expressed in PFU/ml, are given in Table 5 and Fig. 2. Fig. 2 also includes the regression line for vaccine Q, derived from data given by Cockburn et al. (1957).

DISCUSSION

Within the limits of this trial no difference in infectivity is discernible between vaccines L and R. For both, the best estimate of the potency required for a "take" rate of 99% in primary vaccinations was found to be within the limits of 4.0 - 4.5×10^7 PFU/ml, and therefore not far from the recommended minimal level of 5×10^7 PFU/ml.

¹ The 95% confidence limits for the potency ratios were computed according to Fieller (1944) and were also found to be within these dose ratio limits. The ratio of the weighted averages of series 2, 3 and 4 in Table 1 amounted to 2.95 : 1 with 95% confidence limits at 2.70 : 1 and 3.21 : 1. Duplicate tests on the same day in 10 eggs each were taken as one test in 20 eggs in applying Fieller's method, since a 2×2 comparison of four daily tests would involve an arbitrary choice between duplicates. This method seems acceptable inasmuch as the inter-dilution variance components in Table 2 are relatively small.

FIG. 2
PROBIT-PFU-POTENCY REGRESSION LINES
FOR VACCINES L, R AND Q^a



^a Data for vaccine Q are taken from Cockburn et al. (1957).

The differences, as shown in Fig. 2, between our data and the findings of Cockburn et al. for vaccine Q require further comment. There is some, but not a significant, discrepancy in the slope of the regression lines (1.55 and 1.42 respectively), but a more important difference in the 50% effective dose (1.3×10^6 and 3.0×10^5 PFU/ml respectively). This fourfold difference in dose might seem fundamental; nevertheless we hesitate to postulate any serious divergence in the properties of the vaccinia strains. The following points may be of more or less decisive importance in this connexion.

First, the British authors tested a dried vaccine, that "appeared to be a relatively crude preparation", and obtained graded doses by heat inactivation. In our trial, fluid vaccines were used and the desired dosage was obtained by dilution.

Secondly, potency tests on chick embryos performed in two laboratories are not necessarily equivalent; this was also the experience of Cockburn et al. Moreover, the British authors titrated each heat-inactivated subplot of vaccine Q separately, while we concentrated our potency tests on the undiluted products.

Thirdly, vaccination with vaccine Q was done by one 6-mm scratch and with vaccines L and R with two 3-mm scratches. We doubt that this would have any effect on the "take" rate, but slight

differences of technique between two vaccinators might be of importance. Furthermore, although no unfavourable effect of 2 ml of 16% hyperimmune gamma-globulin on the "take" rate is seen when full-potency vaccine is used (Gispen, Lansberg & Nanning, 1956), we have to admit the possibility of some influence on the "take" rates in our trial with vaccines L and R.

Finally, it must be pointed out that Cross, Kaplan & McClean (1958) did a further small-scale trial with heat-inactivated vaccine Q and fluid Lister Institute vaccine and obtained results that were in rather good agreement with our findings:

Vaccine	Potency	"Take" rate
Q	1×10^6	39/99 (39%)
Lister Institute	1.7×10^6	15/39 (37%)

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RÉSUMÉ

Le vaccin antivariolique de l'Institut national de la Santé publique des Pays-Bas (vaccin R) a été comparé, pour contrôle de son efficacité, au vaccin du Lister Institute, de Grande-Bretagne (vaccin L).

L'activité de ces vaccins a été déterminée par numération des pustules sur des groupes de 10 embryons de poulets. Dans 4 séries de 40, 16, 12, et 10 tests, le rapport d'activité vaccin L/vaccin R varia entre 2,6 : 1 et 3,1 : 1. Les variations exogènes dues aux œufs, à la dilution, au jour de l'épreuve, furent de même importance pour les deux vaccins.

L'efficacité des vaccins a été évaluée sur des soldats soumis à leur primo-vaccination, avec des vaccins dilués. Le rapport entre le log de la dilution et le pourcentage

des « prises », analysé par la méthode des probit, était en accord avec le rapport d'activité trouvé par la méthode de numération des pustules. Il n'y a donc pas de différence significative de la relation dose-effet entre le vaccin L et le vaccin R.

Pour les deux vaccins, l'évaluation de la dose efficace à 99% était de $4,0-4,5 \times 10^7$ unités formatrices de pustules, par ml (soit très proche de la quantité 5×10^7 recommandée sur le plan international); la dose efficace à 50% était de $1,3-1,4 \times 10^6$ unités/ml.

L'auteur discute enfin les causes possibles de la différence importante observée, dans la détermination de l'efficacité 50%, entre les vaccins frais L et R d'une part, et le vaccin desséché Q, d'autre part.

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