*Bull. Org. mond. Santé*
*Bull. Wld Hlth Org.* } 1963, **29**, 299-309



Smallpox /n

# The International Reference Preparation of Smallpox Vaccine

## An International Collaborative Assay

P. KRAG, M.D.[1] & M. WEIS BENTZON [2]

*A purified, concentrated sheep vaccine, prepared from a vaccinia strain in use in the United Kingdom for more than 60 years, has been established as the International Reference Preparation of Smallpox Vaccine, intended to permit comparative assay with national reference vaccines.*

*Before its establishment as the International Reference Preparation, the proposed international reference vaccine was tested in seven laboratories in as many countries together with four other distributed vaccines and one local vaccine in each country. All laboratories used the scarification test on rabbits ; four used the pock count method ; the intracutaneous test in rabbits, the plaque count in tissue culture and the $LD_{50}$ test in eggs, cultures and newborn mice were used by one to three of the participants.*

*The proposed international reference preparation met all the requirements laid down by the WHO Study Group on Requirements for Smallpox Vaccine in six laboratories when the scarification test was used and in all laboratories when the pock count test was used.*

An international study on smallpox vaccines was organized in 1959 in accordance with a request of the WHO Expert Committee on Biological Standardization (1959) and following the considerations of a WHO Study Group on Requirements for Smallpox Vaccines (1959).

### MATERIAL AND METHODS

The following seven laboratories participated, one to five tests being performed per laboratory:

School of Hygiene, *Ankara*, Turkey;
Queen Saovabha Memorial Institute, *Bangkok*, Thailand;
Swiss Serum and Vaccine Institute, *Berne*, Switzerland;
Statens Seruminstitut, *Copenhagen*, Denmark;
The Lister Institute of Preventive Medicine, *Elstree*, United Kingdom;

Throughout this report the participating laboratories are referred to by arbitrary numbers, which have no connexion with the above order of listing of the laboratories.

The vaccines distributed were all freeze-dried: sheep vaccine (Lister Institute, Code R and D), calf vaccine (Ecuador, Statens Seruminstitut, Code A), egg vaccines (Berne, Code B, and Moscow, Code C). The proposed international reference preparation was vaccine R.[3] Each laboratory was also asked to include in its assays a local vaccine (Code $L_{1-7}$, of which $L_3$ was an egg vaccine, $L_4$ a sheep vaccine and the others calf vaccines).

The methods for tests and for evaluation are noted in Table 1 with the main abbreviations and the average results for vaccine R.

In the testing plan allowance was made for the

TABLE 1

TESTS AND EVALUATION METHODS USED IN COMPARATIVE STUDY OF SMALLPOX VACCINES

| Test | No. of laboratories in which test was performed | $Log_{10}$ value or titre | Abbreviation | Average value for vaccine R [a] |
|---|---|---|---|---|
| Scarification, rabbit skin | 7 | $Log_{10}$ to number of lesions per area next to confluency | Lesion value: LN | 4.5 [b] (2, 3, 4, 6, 7) |
| Pock count, chorio-allantoic membrane | 4 | Logarithmic pock number per ml vaccine | Pock value: PON | 8.4 (3, 4, 6) |
| Intracutaneous, rabbit skin | 3 | $Log_{10}$ to highest dilution giving a lesion $\geqq$ 6 mm | Intracutaneous titre: IC | 5.2 (6) |
| Tissue culture (KB cells), plaque count | 1 | Logarithmic plaque number per ml vaccine | Plaque value: PLN | 5.8 (2,3) |
| Tissue culture (chick embryo), $LD_{50}$ | 1 | Logarithmic 50 % titre referred to undiluted vaccine | $LD_{50}$ culture | |
| Eggs $LD_{50}$ | 2 | Logarithmic 50 % titre referred to undiluted vaccine | $LD_{50}$ egg | 7.9 (2) |
| Mice $LD_{50}$ | 1 | Logarithmic 50 % titre referred to undiluted vaccine | $LD_{50}$ mice | 6.0 (6) |
| Revaccination of humans | 5 | Weighted percentage of take: vaccinoid (V) and accelerated reactions (AR) $\left(V+\frac{AR}{2}\right)$ | Revacc. | 60 % Lab. 4<br>50 % Lab. 2 and 5<br>40 % Lab. 7<br>20 % Lab. 1 |

[a] Figures in parentheses indicate the laboratories whose results were included in determining the average values.
[b] Per inoculum (0.3 or 0.5 ml); LN per ml about 4.9.

performance of the rabbit test and three days for the other tests:

| *Rabbit test* | | | | *Other tests* | | | |
|---|---|---|---|---|---|---|---|
| 1st day | R | A | L | R | A | B | L |
| 2nd day | R | B | L | R | C | D | L |
| 3rd day | R | C | L | R | C* | D* | L |
| 4th day | R | D | L | | | | |

* Or other of the vaccines distributed.

Major deviations from this plan are noted in the subsequent tables. The relative potencies are calculated as the difference between $\log_{10}$ values or titres for a test vaccine and for the R vaccine tested on same day (rabbit). Average R titres are based on all available results.

For each method a $\log_{10}$ value has been obtained as indicated in Table 1.[1] The $\log_{10}$ results for vaccines B and C were all adjusted (by +0.10) to compensate for the lower content per ampoule (0.20 ml as against 0.25 ml for vaccines R, D and A), as all four vaccines were reconstituted by the same volume of buffer solution. The variances due to the variation within days and between days have been estimated from the variation in the difference R-L and other differences from day to day. For the pock count and the plaque count the relation to the Poisson distribution has been studied and the $\beta$ factor in the expression $s^2 = \bar{x} + \beta \bar{x}^2$ was estimated (see Table 4, notes). Statistical problems regarding the evaluation of the standard deviation for all methods and the basis for the use of $\beta$ factors in the variance for the pock counts are described separately (Bentzon & Krag, to be published).

The standard errors for relative potencies are collected in Table 2 as estimated for the usual number of testing days. The table has a line showing the standard error per method for one experimental

TABLE 2
STANDARD ERROR FOR RELATIVE POTENCIES BY VARIOUS METHODS

| Laboratory | Scarification [a] (n = 2) | Pock count [a] (n = 2) | Intracutaneous [a] | Tissue culture (n = 1) | | Eggs (n = 1) | Mice (n = 1) |
|---|---|---|---|---|---|---|---|
| 1 | 0.12 | | | | | | |
| 2 | 0.29 | | $0.55_4$ | 0.20 | | 0.24 | |
| 3 | 0.31 | 0.10 | | 0.09 | | | |
| 4 | 0.34 | 0.05 | | | | | |
| 5 | 0.21 | | | | | | |
| 6 | 0.31 | 0.07 | $0.33_2$ | | | 0.19 | 0.49 |
| 7 | 0.24 | 0.16 | $0.20_6$ | | | | |
| | | | | Lab. 2 | Lab. 3 | | |
| Usual value for one exp. unit | 0.30<br>1 rabbit | 0.20<br>6 eggs | 0.33<br>1 rabbit | 0.34<br>6 tubes | 0.15 | 0.39<br>6 eggs | 0.78<br>5 mice |

[a] n = the number of experiments for each average titre or relative potency; in Tables 3, 4 and 5 there are values different from the above typical values given as an index. The corresponding standard errors are:

$$SE_n = SE_2 \sqrt{\frac{2}{n}} = SE_1 \sqrt{\frac{1}{n}} .$$

counting methods (0.20 and 0.15) and the $LD_{50}$ in mice (0.78).

## RESULTS

### *Scarification*

The seven laboratories (see Table 3) were in agreement in the general classification of the vaccines: R = D>A>C>B. The LN values for vaccine R varied about 4.7, within a range of 0.95. The range for the test vaccine titres varied from 0.6 to 1.7, while the relative potencies had ranges of 0.8 to 1.2. Vaccines D and A had relative potencies varying about zero with significant deviations only for laboratory 1. Laboratory 5 classified vaccines C and B as equal, with a potency at −0.7; the well-defined results from other laboratories showed for these vaccines a marked difference in potency, the average potency value being −0.5 and −1.5, respectively.

### *Pock count*

The three laboratories (see Table 4) with well-defined results (Laboratories 3, 4 and 6) had R values from 8.0 to 8.6; the ranking of the relative potencies varying about zero with one significant deviation (vaccine A, Laboratory 6). The relative potencies for vaccines C and B had less marked differences than those seen for scarification, the average potencies being −0.8 and −1.4.

### *Intracutaneous test*

The well-defined relative potencies (see Table 5, upper part) for vaccines A and D varied about zero. The relative potencies for vaccines C and B were 0.2 and −1.0 respectively; these differences are similar to those for the scarification test.

### *Tissue culture*

The results (see Table 5) are peculiar owing to the low titres for vaccines R and D. The relative potencies were all above 0.5. Vaccine A had the highest relative potency. The usual difference in relative potency for vaccines A and C was found, but vaccine B gave values close to those for A (Laboratory 2) and for C (Laboratory 3).

### *Eggs $LD_{50}$*

The results (see Table 5, lower part) generally

TABLE 3
AVERAGE TITRES AND RELATIVE POTENCIES FOR THE SCARIFICATION TEST [a]

| Laboratory | Titres | Relative potencies [b] | | | | |
|---|---|---|---|---|---|---|
| | R | D | A | C | B | L |
| 1 | $5.21_4$ | *—0.25 | ***—$0.62_1$ | **—$1.08_1$ | ***(—0.87) [c] | —$0.43_4$ |
| 2 | $4.92_8$ | 0.09 | 0.48 | (—0.68) | —1.08 | $0.06_8$ |
| 3 [d] | $4.45_8$ | 0.48 [e] | >0.24 | —0.38 | —1.33 | —$1.13_8$ |
| 4 | $4.78_8$ | 0.05 | —0.47 | —0.49 | —$1.90_4$ [e] | $0.00_8$ |
| 5 | $4.90_8$ | 0.08 | —0.23 | —0.72 | **—0.75 | —$0.09_8$ |
| 6 | $4.31_{14}$ | $0.01_4$ | $0.20_4$ | —$0.52_4$ | —1.99 | $0.00_{14}$ |
| 7 [f] | $4.26_8$ | —0.28 | 0.10 | —0.23 | —1.26 | —$1.47_8$ |
| Range of titres | 0.95 | 0.92 | 1.32 | 0.61 | 1.74 | |
| Range of rel. pot. | | 0.76 | 1.10 | 0.85 | 1.24 | |
| Aver. rel. pot. | | 0.03 | —0.04 | —0.59 | —1.31 | |

[a] The index indicates the value for n (number of testing days) if different from the usual number for scarification, i.e., 2.
[b] The signs *, **, *** indicate that the relative potency lay outside the limits of significance (P = 5, 1 and 0.1 % respectively). Relative potencies in parentheses are based on less reliable readings, one or more ≦ 2.
[c] Relative potency estimated as B-L + $\overline{\text{L-R}}$.
[d] Laboratory 3 titres were estimated as scarification titres (highest dilution with confluency) and corrected to lesion-values (see Table 1) by addition of log dilution factor + log average count (0.48 + 0.30 = 0.78).
[e] Relative potencies based on results from flat and/or irregular curves.
[f] Majority of dose-response curves flat or irregular.

therefore relative potencies were based on the results for vaccine L.

*Mice $LD_{50}$*

The results (see last line, Table 5) differed from the results of all the other methods in having extremely low relative potency for vaccine A (—2.9) while vaccines C and B were of the same magnitude (about —0.4).

*Local vaccines*

According to the assay plan each of the seven local vaccines (see also Table 8) was examined only in the laboratory where it was known. $L_1$, $L_2$, $L_4$, $L_5$ and $L_6$ had relative potencies of the same magnitude as those for vaccines D and A, while vaccines $L_3$ and $L_7$ in scarification and pock count followed vaccine B, but $L_7$ by the intracutaneous test had a high potency of 0.5 (vaccine B: —1.0).

*Revaccination in humans*

immune reaction), " AR " (accelerated reaction) and " V " (vaccinoid reaction): the values 0 and 1 were assigned to the qualities I and V; AR representing an intermediate result was arbitrarily given the value ½. In this way it was possible to estimate an " average lesion " for each vaccine. The index varied between zero (all persons having the result I) and 1.0 (all persons having the vaccinoid lesion).

The comparison between the reference vaccine and the test vaccine was performed indirectly, i.e., through the results relative to the L vaccines, as shown in the following example:

For Laboratory 4, Vaccine C, indices were based on the results from two groups of revaccinated humans, at least 25 persons in each; one group was vaccinated with R and L, the other with C and L:

| | *Vaccines* | | *Vaccines* | |
|---|---|---|---|---|
| | *R* | *L* | *C* | *L* |
| I | 8 | 7 | 39 | 20 |
| AR | 60 | 57 | 48 | 55 |
| V | 32 | 36 | 13 | 25 |
| Index * | 0.63 | 0.64 | 0.37 | 0.53 |

TABLE 4
AVERAGE TITRES AND RELATIVE POTENCIES FOR THE POCK COUNT IN EGGS

| Laboratory | Vaccines [a] | | | | | |
|---|---|---|---|---|---|---|
| | R | D | A | C | B | L |
| 3 | $8.00_3$ | 0.00 | $-0.53_1$ | −0.73 | $-1.33_1$ | $-1.55_3$ |
| 4 | $8.59_3$ | $-0.04_1$ | $-0.33_1$ | $-1.01_1$ | $-1.58_1$ | $-0.11_3$ |
| 6 | $8.55_5$ | $-0.03_3$ | *−0.17 | $-0.78_3$ | −1.29 | $-0.43_5$ [b] |
| 7 | $>8.13_2$ [c] | $>0.54_1$ | $(<-0.64)_1$ | $-0.51_1$ | $(<-1.51)_1$ | $(<-1.23)_2$ |
| Lab. 3, 4, 6 | | | | | | |
| Range of values | 0.59 | 0.57 | 0.94 | 0.48 | 0.63 | |
| Range of relative potency | | 0.04 | 0.36 | 0.28 | 0.29 | |
| Average relative potency | | −0.02 | −0.34 | −0.84 | −1.40 | |

[a] Titres in parentheses are based on average pock count ≦ 9.0.
[b] 12 out of 26 membranes with less well defined (but counted) lesions.
[c] About 50 % of membranes without lesions (vaccines R, D, B and L: 4/11, 2/4, 3/6 and 7/12 respectively).

Pock count: value of $\beta$ in $s^2 = \bar{x} + \beta \bar{x}^2$ and corresponding $s_W$-values:

| Laboratory | Range of average pock counts | Estimated value of $\beta$ | Estimate of standard deviation ($s_W$) |
|---|---|---|---|
| 3 | 4-18 | 0.225 | 0.097 |
| 4 | 10-21 | 0.049 | 0.064 |
| 6 | 8-21 | 0.073 | 0.067 |
| 7 | 1-29 | 1.413 | 0.220 |

It is only under the presumptions: (*a*) that the dose-index curves for vaccines R and L are parallel, and (*b*) that the curves are approximately linear over the dose interval used, that the index difference will give an estimate of the quantity " potency of R relative to C multiplied by the common slope of the dose-index curves ".

The well-defined index differences (see Table 6) obtained for Laboratories 2 and 4 classified the vaccines D = A>C>B; the other three laboratories gave results with similar classification as above, but the high percentage of takes made the comparison less accurate. The differences in takes between laboratories correspond tolerably well to the differences in distribution for the five groups of population in the interval between primary vaccination and this revaccination.

*Precision of the methods*

between two vaccines, a reference vaccine R and a local vaccine L. For a given true difference in *scarification*, $\delta$, the number of experiments required, $n$, to have 95 % probability of observing a difference significant at the 5 % level was:

| $\delta =$ | 0.3 | 0.6 | 0.9 | 1.2 |
|---|---|---|---|---|
| $n =$ | 25 | 7 | 3 | 2 |

For the *pock count* the corresponding figures were:

| $\delta =$ | 0.1 | 0.2 | 0.3 |
|---|---|---|---|
| $n =$ | 16 | 4 | 2 |

The statistical method is described elsewhere (Bentzon & Krag, to be published).

It is concluded that differences smaller than 0.40 cannot be demonstrated by scarification without

TABLE 5
VARIOUS TESTS

| Laboratory | Titres | Relative potencies | | | | |
|---|---|---|---|---|---|---|
| | R | D | A | C | B | L |
| Intracutaneous | | | | | | |
| 2 | $4.10_{10}$ | $0.25_4$ | $-0.50_4$ | $0.10_4$ | $-0.90_4$ | $-0.10_{10}$ |
| 6 | $5.18_8$ [a] | $\geqq -0.30_2$ [a] | $0.30_2$ [a] | $\geqq -0.31_2$ [a] | $-0.91_2$ [a] | $-0.15_8$ [a] |
| 7 [b] | | $0.02_6$ | $-0.11_6$ | $0.40_{12}$ | $-1.08_6$ | $0.56_{18}$ |
| Tissue culture | | | | | | |
| 2 | $5.50_3$ | $0.08_1$ | $1.40_1$ | $0.58_1$ | $1.26_1$ | $0.45_3$ |
| 3 | $6.13_3$ | $>-0.02_2$ | $1.23_1$ | $0.69_2$ | $0.51_1$ | $0.22_3$ |
| Egg $LD_{50}$ | | | | | | |
| 2 | $7.93_3$ | $-0.48_1$ | $0.06_1$ | $-0.38_1$ | $-0.80_1$ | $0.05_3$ |
| 2 [c] | | $-0.48_2$ | $0.08_1$ | $-0.54_1$ | $-0.79_1$ | |
| 6 [c] | $7.79_3$ [a] | | $-0.18_1$ | $-0.78_1$ | $-1.15_1$ | |
| Mice $LD_{50}$ | | | | | | |
| 6 [d] | $6.00_1$ | | $-2.88_1$ | $-0.45_1$ | $-0.32_1$ | $1.38_1$ |

Index indicates number of testing days.
[a] Flat or irregular dose-response curves.
[b] Relative potency estimated directly from well-defined dose-response curve.
[c] Relative potency based on L values.
[d] Relative potency for vaccines C and L was based on vaccine D (instead of vaccine R).

TABLE 6
THE RELATIVE INDICES AND INDEX DIFFERENCES [a] PER LABORATORY

| Laboratory | $(I_R - I_L)$ [b] | $(I_T - I_L) - (\overline{I_R - I_L})$ [b] | | | | Average |
|---|---|---|---|---|---|---|
| | Vaccine R | Vaccine D | Vaccine A | Vaccine C [c] | Vaccine B [c] | A, C, B |
| 1 | 0.12 | —0.19 | —0.27 | (—0.20) | (—0.12) | (—0.20) |
| 2 | —0.03 | 0.00 | 0.00 | —0.20 | —0.42 | —0.21 |
| 4 | —0.01 | 0.00 | —0.02 | —0.15 | —0.32 | —0.16 |
| 5 | 0.50 | 0.05 | (—0.23) | (—0.50) | (—0.45) | (—0.39) |
| 7 | 0.16 | 0.04 | —0.26 | —0.51 | (—0.29) [d] | —0.35 |
| Average difference | | —0.02 | —0.16 | —0.31 | —0.32 | —0.26 |

[a] Values in parentheses indicate less well defined index differences.
[b] $I_R$, $I_L$ and $I_T$ = the index for vaccines R, L and a test vaccine:
The relative index $I_T - I_L$ is the difference between indices of the test vaccine and the corresponding local vaccine.
The relative index $I_R - I_L$ is the difference between indices of the proposed reference preparation and the corresponding local vaccine.
The index difference $(I_T - I_L) - (\overline{I_R - I_L})$ is the difference between the relative indices of the test vaccine and the proposed

*Minimum requirements*

The published report of the WHO Study Group on Requirements for Smallpox Vaccine (1959) stipulated results to be achieved in potency tests by scarification, pock count and determination of $LD_{50}$ in eggs. The proposed reference vaccine passed these requirements in most of the laboratories:

| *Scarification* | *Pock count* | *Eggs $LD_{50}$* |
|---|---|---|
| 8/8 tests: Lab. 1, 2, 4 | Passed in all | 2/4 tests: Lab. 2 |
| 7/8 tests: Lab. 5,7 | laboratories | 2/5 tests: Lab. 6 |
| 6/8 tests: Lab. 3 | | |
| 9/16 tests: Lab. 6 | | |

Vaccine A also passed in most tests, but vaccines C and B failed as a rule.

The present wording of the requirements creates some problems as it states that in potency tests in the scarified skin of rabbits *both* vaccines—reference vaccine (R) and the vaccine under test (T)—should produce the specified lesions. In some cases both vaccines may pass the requirements but the R vaccine may give much larger lesions than the T vaccine; these results may reflect extra-large responses in a sensitive rabbit under test, and in a series of other rabbits only vaccine R may pass. Conversely, in cases where the T vaccine just passes while the R vaccine does not, a series of repeated experiments may show that the R vaccine just passes while the T vaccine produces large lesions. Therefore it may be preferable to base the acceptance criteria on potencies estimated on titres in the usual way rather than on passing an arbitrary limit in an animal test.

The requirements for potency tests by determination of $LD_{50}$ and by pock count after application of vaccine to the chorio-allantoic membrane of chick embryos specify results to be obtained corresponding to $\log_{10}$ values of 7.85 and 7.70, respectively; these two requirements are in accordance, if one pock-forming unit has the ability to kill an egg, since an average of 0.7 unit per volume inoculated corresponds to a 50% chance of introducing 1 unit or more in the eggs. On this assumption the expected difference between the logarithmic titres is 0.15.

Only laboratory 6 used pock count as well as $LD_{50}$; it will be seen that vaccines R, D and A passed the pock-count minimum requirements, while in the $LD_{50}$ test the R vaccine passed in less

| | *Vaccines* | | | | | |
|---|---|---|---|---|---|---|
| | *R* | *D* | *A* | *C* | *B* | *L* |
| Pock count | 8.55 | 8.50 | 8.40 | 7.75 | 7.29 | 8.12 * |
| $LD_{50}$ | 7.79 ** | 7.83 ** | 8.26 | 7.88 | 7.28 | 8.45 |
| Difference | −0.76** | −0.67** | −0.14 | 0.13 | −0.01 | 0.33 * |

* Value based on readings including less well defined pocks.
** Value based on flat or irregular dose-response curves.

Vaccines R and D have a low relative $LD_{50}$, with an average difference of −0.72; vaccines A, C, B and L have a high relative $LD_{50}$, with an average difference of 0.08, while the expected difference was 0.15.

Vaccines R and D (produced in sheep) are not related to the other vaccines.

The observations may be explained by the hypothesis that R has a low virulence for eggs, but the flatness and irregularity of the dose-response curves for R (and D) should be remembered, as these titres are badly defined.

Further tests may be needed to clarify whether the $LD_{50}$ in eggs generally is a more severe test or whether some vaccines have a low $LD_{50}$ value owing to lower virulence.

## DISCUSSION

Reviewing the above details on the results for the seven groups of methods (see Table 7), it is seen that the results of testing the potency of the vaccines by revaccination, scarification and pock count tests usually agreed. The other four groups of methods were examined only in four laboratories and with one to three laboratories per group.

The major deviations are listed below for each vaccine.

The *R vaccine* gave rather low values in the $LD_{50}$ test in eggs.

The *A vaccine* gave (relative to R) high values in the tissue culture test, but extremely low values in the $LD_{50}$ test in mice, while the *B vaccine* had rather high values in the tissue culture test as well as in the $LD_{50}$ test in mice. In the revaccination test vaccine A showed differences in relative index from population to population.

The *C vaccine* gave a high value in the intracutaneous test.

Of the local vaccines, $L_2$ showed low values in the tissue culture test, $L_4$ low values in the $LD_{50}$ test

TABLE 7
AVERAGE RESULTS BY DIFFERENT METHODS FOR EACH VACCINE

| Test method | Evaluation method | Laboratory | Vaccines | | | |
|---|---|---|---|---|---|---|
| | | | D | A | C | B |
| Revaccination (humans) | Index | 2 | 0 | 0 | —0.20 | —0.42 |
| | difference | 4 | 0 | —0.02 | —0.15 | —0.32 |
| Scarification (rabbit) | Average of relative potency | 2, 3, 4, 6, 7 | 0.07 | 0.11 | —0.46 | —1.51 |
| Pock count (egg) | | 3, 4, 6 | —0.02 | —0.34 | —0.84 | —1.40 |
| Intracutaneous (rabbit) | | 6, 7 | —0.14 [a] | 0.10 [a] | —0.04 [a] | —1.00 [a] |
| Tissue culture ($LD_{50}$, plaques) | | 2, 3 | 0.05 | 1.32 | 0.64 | 0.89 |
| $LD_{50}$ egg | | 2 | —0.48 | 0.06 | —0.38 | —0.80 |
| $LD_{50}$ mice | | 6 | | —2.88 | —0.45 | —0.32 |

[a] Flat or irregular dose-response curve.

From this it is clear that even with a limited number of vaccines (as in this assay) differences were seen when several methods and groups were used, and the disagreements found underline the necessity for intensive studies to define the extent and the practical value of these findings.

## EFFECT ON POTENCY DETERMINATION OF USE OF PROPOSED REFERENCE VACCINE

From the material in this report it is seen that the use of a reference vaccine has been to a varying degree successful for the comparison of results.

*Same method, same laboratory*

Pock count results showed in Laboratories 3 and 6 that the variation of the difference between pock values (R-L) exceeded that expected from the estimated variance; other variations in the difference R-L within laboratories noted are in the scarification test in Laboratories 3, 4 and 6, and in the intracutaneous test in Laboratory 2. This showed that vaccine R and some of the local vaccines gave results which did not vary in the same way under all conditions.

*Same method, different laboratories*

The range of potencies relative to vaccine R per method was generally lower than the corresponding —scarification: vaccine R, Laboratory 1, and vaccine B, Laboratories 1 and 5; pock count: vaccine A, Laboratory 6; tissue culture: vaccine B.

*Different methods, same laboratory*

Potency differences have been calculated for certain pairs of methods used in the same laboratory (see Table 8). Here we have the possibility of examining the potency differences for various vaccines, including some of the local vaccines ($L_{1\text{-}7}$) tested in each individual laboratory. The ideal value of such potency differences is zero with variations within the range of the general variation for potency differences.

Two other types of potency differences were observed:

1. Potency differences varied about an average value—e.g., −0.40 for vaccines A, C, B and L. This indicated that the variations from method to method for those four vaccines followed the same pattern but this differed from the variation in titre level for vaccine R; in other words, vaccine R was not useful as a reference for these four vaccines tested with this pair of methods.

2. Potency differences varied from vaccine to vaccine—e.g., vaccine A, difference −0.14; vaccine D, difference +0.32; vaccine B, difference +0.76. Potency differences of that sort indicated that

TABLE 8

COMPARISON OF RELATIVE POTENCIES FOR DIFFERENT METHODS [a]

| Method differences | Vaccines | Laboratories | | | | |
|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 6 | 7 |
| PON — LN | ACB | | <—0.30 | 0.05 | 0.09 | (<—0.35) |
| | L | | *—0.42 | —0.11 | ***—0.43 [b] | <0.24 [c] |
| IC — LN | ACB | 0.06 | | | >0.53 [c] | 0.27 |
| | L | —0.16 | | | —0.15 | ***2.03 |
| TC [d] — LN | ACB | ***1.58 | ***<1.37 | | | |
| | L | *0.39 | ***1.35 | | | |
| Egg — LN | ACB | 0.12 | | | 0.09 [e] | |
| | L | —0.01 | | | | |
| Mice [f] — LN | ACB | | | | —0.38 | |
| | L | | | | **1.38 | |
| PON — IC | ACB | | | | *—0.37 | * (<—0.55) |
| | L | | | | —0.28 [b, e] | ***<—1.79 |
| PON — TC | ACB | | ***—1.60 | | | |
| | L | | ***—1.77 | | | |
| PON — Egg | ACB | | | | **0.44 | |
| | L | | | | **0.33 [b, e] | |
| PON — Mice | ACB | | | | 0.54 | |
| | L | | | | ***—1.81 | |

| | Average ACB values for the methods: | | | | | |
|---|---|---|---|---|---|---|
| | LN | PON | IC | TC | Egg | Mice |
| Titre | 3.94 | 7.54 | 4.71 [g] | 6.69 | 7.51 | 4.96 |
| Relative potency | —0.62 | —0.86 | —0.28 [c] | 0.95 | —0.37 | —1.21 |
| Laboratories | 2, 3, 4, 6, 7 | 3, 4, 6 | 6, 7 | 2, 3 | 2 | 6 |

[a] Asterisks (*, **, ***) refer to SE for relative potencies for one experimental unit (see Tables 2 and 3). Values in parentheses are based on pock count ≦ 9.0.

[b] Less well defined pock count included.

[c] Flat or irregular dose-response curve.

a reference for vaccines A, L and maybe C, but not for vaccine B (using that pair of methods).

It may be noted that potency differences following the latter pattern were mostly seen when methods were compared with the scarification results, while such differences were not seen when the basis for the comparison was the pock count, in which case constant potency differences were found for pairs of methods.

The conclusion is that all methods were in agreement regarding the low potency of vaccine B, but vaccine B was in addition less able to produce lesions in the skin of rabbits.

It is unlikely that a reference vaccine would serve as a useful reference for *all* vaccines regardless of the method used; on the other hand, a reference vaccine such as vaccine R may be of importance other than for equalizing results as it is of value to classify the vaccines in groups according to the potency difference (between methods).

The classification (Table 7) of the vaccines was made from the revaccination results: vaccines R and A were more potent than vaccines C and B, the latter showing the lowest effect, corresponding to the average results for two of the laboratory methods (scarification and pock count), while the other methods, which were used in one to three laboratories only, gave deviating relations between potencies for one or more vaccines per method.

As the variation between rabbits tested with the same vaccine was more pronounced than the variation on pock count, the latter method is to be recommended.

## THE INTERNATIONAL REFERENCE PREPARATION AND ITS USE

The International Reference Preparation of Smallpox Vaccine, which was established in 1962 by the WHO Expert Committee on Biological Standardization (1963), is a purified, concentrated sheep vaccine prepared from a vaccinia strain used in the United Kingdom for more than 60 years. In recent years it has been passed alternately in rabbits and sheep. The supernatant from centrifugation at 1500 r.p.m. of the virus-containing pulp, suspended in McIlvaine sodium-phosphate/citric-acid buffer, was centrifuged at 10 000 g after incubation for 48 hours at 22°C. The sediment dissolved in McIlvaine buffer was diluted in 5.5% peptone solution to give a pock count on eggs of $10^{8.7}$.

The suspension was distributed on ampoules with 0.25 ml in each, freeze-dried, filled with oxygen-free $N_2$, and flame-sealed.

The vaccine met all requirements of the United Kingdom's Therapeutic Substances Act, 1956.

The freeze-dried vaccine was unchanged in pock count after storage at 37°C for one month and at 4°C for at least 13 months.

Ampoules of the International Reference Preparation were tested as vaccine R (and D) in the international assay described above. The International Reference Preparation is intended for comparison with national reference vaccines.

### *The use of the reference*

The full content of one ampoule dissolved in 2.5 ml of McIlvaine buffer gives a vaccine dilution of 1: 10. The average strength of the vaccine, as found in the assay, was:

| | | |
|---|---|---|
| Pock count on chorio-allantoic membrane about | $10^{8.4}$ | per ml |
| Scarification, lesion value | $10^{4.9}$ | „ „ |
| Intracutaneous test | $10^{5.2}$ | „ „ |
| $LD_{50}$ in eggs | $10^{7.9}$ | „ „ |
| $LD_{50}$ in tissue culture | $10^{5.8}$ | „ „ |
| $LD_{50}$ in newborn mice | $10^{6.0}$ | „ „ |

## RÉSUMÉ

Un vaccin de mouton purifié et concentré, préparé à partir d'une souche de vaccine utilisée au Royaume-Uni depuis plus de 60 ans, a été choisi comme Préparation internationale de référence de vaccin antivariolique.

Avant d'être désigné comme Préparation internationale de référence, ce vaccin (vaccin R) a été testé dans sept laboratoires, celle de la numération des pustules sur membrane chorio-allantoïdienne de poulet par quatre d'entre eux, celle des injections intradermiques au lapin par trois, celle de la numération des pustules en culture de tissus (cellules KB) par un, celle du calcul de la $DL_{50}$ en culture de tissus

ont procédé à des revaccinations humaines avec établissement du pourcentage de réactions vaccinoïdes et de réactions accélérées.

Les variations entre les résultats du test de scarification sur le lapin ont été plus importantes que celles du test de numération des pustules. Le titre logarithmique des lésions a été en moyenne de 4,5 par la méthode de scarification et de 8,4 par celle de la numération des pustules. Le classement des vaccins obtenu en comparant leur activité à celle du vaccin R a été presque le même avec les deux méthodes et la moyenne des activités relatives pour les vaccins codés A (vaccin de veau utilisé en Equateur et au Statens Seruminstitut de Copenhague), B (vaccin sur œuf, de Berne) et C (vaccin sur œuf, de Moscou) a été respectivement de 0,1, −0,7 et −1,5.

Les autres tests ont mis en évidence pour un ou plusieurs vaccins des valeurs déviant des précédentes. Les discordances suivantes sont à noter: le titre du vaccin R dans le test de culture de tissus est bas; avec le vaccin A, l'on obtient une activité relative très faible dans le test $DL_{50}$ sur souriceaux nouveau-nés.

Dans plusieurs cas, les sept vaccins de fabricati locale ont accusé des différences d'activité relative d' test à l'autre.

Cinq laboratoires se sont livrés à une étude des rev cinations. L'on a noté un certain rapport entre résultats du test et le laps de temps écoulé entre dernière vaccination et la revaccination. La répo obtenue avec les vaccins distribués a correspondu a résultats des tests de scarification et de numération pustules; les vaccins R et A sont d'activité identiq les vaccins C et B sont moins actifs.

Le vaccin R a satisfait aux critères énoncés par Groupe OMS d'étude des Normes relatives au Vac antivariolique dans six laboratoires lors de l'utilisati du test de scarification et dans tous les laboratoires l de l'utilisation du test de numération des pustules.

L'on prévoit que lors d'une revision des crité actuels, les spécifications comprendront un test quar tatif de la Préparation de référence internationale, a une limite d'activité au-dessous de laquelle un vaccin peut être utilisé chez l'homme.

## REFERENCES

World Health Organization, Expert Committee on Biological Standardization (1959) *Wld Hlth Org. techn. Rep. Ser.*, **172**, 12

World Health Organization, Expert Committee on Biological Standardization (1961) *Wld Hlth Org. techn. Rep. Ser.*, **222**, 16

World Health Organization, Expert Committee Biological Standardization (1963) *Wld Hlth O techn. Rep. Ser.*, **259**, 17

World Health Organization, Study Group on Requi ments for Smallpox Vaccine (1959) *Wld Hlth O techn. Rep. Ser.*, **180**