VII. Validation of an Alternative Method to Animal Challenge Assay for Diphtheria Vaccine: Outcome of a Collaborative Study

Project Leader: Dr. A.M. Gommer
RIVM Laboratory for Medicines and Medical Devices
The Netherlands

AIMS OF THE STUDY

The collaborative study described below was organised in 1992-94 for the testing and possible validation of an alternative assay to the current diphtheria potency tests required by the Ph. Eur. monograph (2.7.6).

The collaborative study consisted of a parallel potency assay of the diphtheria toxoid component in a number of different vaccines, using the European Biological Reference Preparation (Ph. Eur. BRP) as reference.

The study included three test methods: intradermal challenge in guinea-pigs, lethal challenge in guinea-pigs and a test based on the detection of neutralising antibodies in the sera from mice immunised with the vaccines to be tested (Vero cell assay).

This report is divided into two parts. In Part 1, the main study, seventeen laboratories were involved. In this study, as regards the vero cell assay, results did not lead to a clear conclusion. Therefore, it was decided to initiate a follow-up study, the results of which are presented in Part 2.

1 — Main Study

PARTICIPANTS

Seventeen laboratories (11 OMCLs and 6 manufacturers) from eleven different countries participated in the study, and are listed at the end of the report. The code number used in this report to identify participants does not correspond to the order of listing.

MATERIAL AND METHODS

Materials

Vaccines

The vaccine used as a reference in the study was the Ph. Eur. BRP Diphtheria vaccine (adsorbed), (ref. no. D2700000). This reference was manufactured by Behringwerke (Germany), each ampoule containing 75 Lf diphtheria-toxoid. It has an assigned a potency of 140 IU per ampoule[1].

Several commercial vaccines, DT-vaccine, DTP-vaccine and D-vaccine, were included in the study and coded A, B and C, respectively. A fourth DT vaccine of inferior quality, coded D, was specially prepared by incubating a (non-commercial) batch of DT-vaccine for 4 hours at 56 °C.

In the table below the manufacturers' information on the potencies (IU per ml), the diphtheria-toxoid content, expressed in *Limes floculatae* (Lf) units, and the aluminum content of the different vaccine samples is given. In the study the vaccines were coded as indicated in the table.

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(1) Mussett M.V., Sheffield F. (1973). *Journal of Biological Standardization* 1, 259-283.

The claimed potencies of the samples A and B are based on the results of one intradermal challenge assay, performed according to the method of the European Pharmacopoeia. The claimed potency of sample C is based on the results of one lethal challenge assay, performed according to the method of the European Pharmacopoeia. The claimed potency of sample D is based on the result of one Vero cell assay (Kreeftenberg method; see below), before heat-treatment.

SAMPLE	CODE	CLAIMED POTENCY (IU/ml) and 95 per cent Confidence Limits	Lf - CONTENT (per ml)	Aluminum content (per ml)
Reference	coded; original label	46.67	25	1.5 mg* Al(OH)$_3$
DT-Vaccine	A	94 (68 - 132)	50	3.4 mg Al(OH)$_3$
DTP-Vaccine	B	140 (100 - 196)	50	3.6 mg Al(OH)$_3$
D-Vaccine	C	113 (84 - 151)	50	3.2 mg AlPO$_4$
DT-Vaccine of inferior quality	D	25 (14 - 43)	5	3.0 mg AlPO$_4$

*after reconstitution in 3 ml.

Diphtheria toxin

Ampoules containing 1000 Lf of freeze-dried diphtheria toxin (Dt 79/1) provided by the RIVM were used. The participants were instructed to reconstitute the diphtheria toxin with 20 ml saline to obtain a concentration of 50 Lf per ml. After reconstitution, the toxin was stored at − 20 °C.

Diphtheria antitoxin

In the study the First International Standard for Diphtheria Antitoxin (equine), provided by the World Health Organisation (WHO) was used. The antitoxin concentration of this solution is 10 IU antitoxin per ml.

Control antiserum

Each participant received 1.5 ml antiserum provided by the RIVM to be used in the alternative potency test as an assay control. At the RIVM 100 mice were immunised subcutaneously with 0.5 ml of a dilution of the Ph. Eur. Diphtheria reference corresponding to 4 Lf per ml. Five weeks (35 days) after this immunisation the mice were bled, the blood pooled and successively incubated for two hours at 37 °C and two hours at + 4 °C. Blood was then centrifuged for 20 minutes at 800 g. The serum was siphoned off, inactivated for 30 minutes at 56 °C and stored at − 20 °C.

Vero cells

Each participant of the study received one vial, containing 1 ml of a frozen suspension of Vero cells at 4×10^6 cells per ml. Most laboratories did not succeed in culturing these Vero cells and used their own Vero cells for this study.

Experimental animals

Participants performing a lethal challenge were asked to use guinea-pigs with a body-weight of 250 to 350 g. Participants performing the alternative test method were asked to use mice, male or female with a body-weight of 10 to 14 g.

Method

Study design

The study included three test methods:
- intradermal challenge in guinea-pigs (in conformity with Ph. Eur. 2.7.6.)
- lethal challenge in guinea-pigs (in conformity with Ph. Eur. 2.7.6.)
- an alternative potency test based on the detection of neutralising antibodies in the sera from mice immunised with the vaccines to be tested (Vero cell assay).

For the Vero cell assay, the participants were asked to perform the test in conformity with the 'Kreeftenberg method'[1] following a protocol drafted by the project leader.

Other methods could also be included. Three participating laboratories performed the Vero cell assay in conformity with the 'Aggerbeck method' in which the visual assessment of the end point is replaced by a spectrophotometric measurement[2].

One of the participating laboratories performed the Vero cell assay assessing the end point by adding a thiazolyl blue staining solution to the tissue culture plates ('Sesardic method').

The participants were asked to perform each assay method two times, on two separate occasions.

RESULTS

Three participating laboratories performed the lethal challenge assay and three other laboratories performed the intradermal challenge assay. Seventeen laboratories performed the Vero cell assay in conformity with the 'Kreeftenberg method'. One of the laboratories tested the reference vaccine and the samples independently from each other, i.e. both the immunisations and the antitoxin titration were carried out on separate occasions. The scores of this laboratory were left out from further calculations.

Estimated potencies

The potencies of the samples A, B, C and D were calculated on basis of the data obtained by means of the various methods.

The potencies based on the lethal challenge data were calculated by probit analysis. All valid results are tabulated below.

Table 1. — *Results of the lethal challenge*

Laboratory	Sample A	Sample B	Sample C	Sample D
4	** *	** *	** 99 (56-71)	* *
7	102 (53 - 163) not done	137 (81 - 218) not done	* 78 (45-131)	32 (16 - 37) not done
11	55 (19 - 91) 50 (23-81)	221 (138 - 443) *	86 (56 - 125) 70 (47-99)	* *

(Estimated potencies with 95 per cent confidence limits: IU/ml).
* ED50 values outside dose-response curve.
**No dose-response curve (vaccine doses too low).

The potencies based on the individual guinea-pig- scores in the intradermal challenge test were calculated by parallel line analysis, after the frequency distribution of each treatment group was checked for deviations from normality. All valid results are tabulated below.

Table 2. — *Results of the intradermal challenge*

Laboratory	Sample A	Sample B	Sample C	Sample D
1	* 90 (64 - 126)	** 154 (84 - 249)	** 54 (30 - 102)	** **
5	121 (78 - 197) 106 (68 - 175)	200 (138 - 316) 195 (115 - 402)	67 (47 - 92) 112 (72 - 184)	** 3.0 (0.8 - 6.0)
13	* *	285 (225 - 360) 205 (131 - 293)	** **	** **

(Estimated potencies with 95 per cent confidence limits: IU/ml).
* No significant linearity (p < 0.05).
** No dose response curve (vaccine doses too low).

(1) Keeftenberg J.G., van der Gun J., Marsman F.R., Sekhuis V.M., Bhandari S.K., Maheshwari S.C. (1985). *Journal of Biological Standardization* **13**, 229-234.
(2) Aggerbeek H. and Heron I. (1991). *Biologicals* **19**, 71-76.

In general, immunisation with the various dilutions of sample D did not result in a dose-response curve as a consequence of a too low immunising potency of the sample.

The potencies based on the Vero cell assay data (Kreeftenberg method) were calculated by parallel line analysis. All valid results are tabulated below. The non-valid assays deviated either from parallelism or from linearity.

Table 3. — *Results of the Vero Cell assay*

Laboratory	Sample A	Sample B	Sample C	Sample D
1	-	-	-	
	60 (31 - 101)	307 (157 - 981)	48 (26 - 79)	-
2	-	-	28 (11 - 50)	-
	26 (6 - 54)	-		-
3	97 (44 -223)		115 (74 - 191)	-
	82 (45 - 145)	607 (329 - 1643)	93 (57 - 152)	-
4	-	-	-	-
	60 (41 - 85)	-	82 (51 - 129)	-
5	-	95 (59 - 153)	73 (46 - 110)	-
	63 (25 - 130)	-	123 (67 - 248)	-
6	43 (20 - 75) -	-	-	-
		-	-	-
7	66 (30 - 126) -	-	82 (24 - 243)	-
	-	-	-	-
8	-	-	-	-
	-	819 (320 - 7447)	-	-
9	49 (32 - 70)	-	63 (42 - 90)	-
	-	-	-	-
10	221 (137 - 409)	-	-	-
	-	-	114 (79 - 162)	-
11	74 (50 - 107)	-	-	-
	76 (55 - 105)	-	-	-
12	-	-	-	-
	180 (123 - 287)	-	-	-
13	80 (49 - 127)	245 (141- 553)	89 (59 - 134)	-
	-	-	-	-
14	-	-	-	-
	75 (22 - 135)	-	112 (79 - 163)	-
15	62 (22 - 135)	650 (303 - 2827)	87 (37 - 202)	1.3 (0.0 - 4.6)
	88 (54 - 145)	378 (184 - 1432)	-	-
17	-	-	-	-
	-	-	140 (67 - 380)	-

(Estimated potencies and 95 per cent confidence limits: IU/ml).

Spectrophotometric measurement of colour formation (Aggerbeck method)

Three participating laboratories performed the Vero cell assay in conformity with the 'Aggerbeck method'. From the data of one of these laboratories it was not possible to calculate valid potencies due to poor dose-response curves. The data of the other two laboratories are tabulated below.

Table 4. — *Results of the Aggerbeck method*

Laboratory	Sample A	Sample B	Sample C
9	36 (24 - 51)	201 (135 - 333)	44 (30 - 61)
	36 (26 - 47)	160 (125 - 213)	48 (34 - 63)
15	69 (28 - 166)	523 (255 - 1075)	85 (30 - 236)
	69 (32 - 148)	512 (177 - 1481)	62 (27 - 142)

(potencies and 95 per cent confidence limits: IU per ml)

There was no significant difference between the potencies of the samples estimated by means of the Kreeftenberg method and those estimated by means of the Aggerbeck method. However there is a significant difference between the potency of sample B as estimated by laboratory 9 and laboratory 15 (*t*-test: p = 0.04).

MTT cytotoxicity assay (Sesardic method)

The results obtained by means of the MTT cytotoxicity assay, performed by one of the participants (laboratory 6) did not differ from the results of the Kreeftenberg method. This means that the end point assessed by adding MTT solution to the microtiterplates in order to detect viable cells, did not essentially differ from the end point assessed on basis of colour change of the culture medium. Hence the equivalence point corresponded with an absorbance value of approximately 0.5 read at 570 nm, negative wells having an absorbance value of less then 0.5 and positive wells having an absorbance value of more than 0.5.

Using the optical density data, participant 6 estimated the relative activity of each mouse serum, i.e. relative to within-plate reference serum (First International Standard for Diphtheria Anti-toxin). With these relative activity estimates of the mouse sera, the potencies of the coded samples A, B, C and D were calculated relative to the reference vaccine, the potency of which was taken as 1.

Calculated this way, no valid estimates were obtained for sample B. However, using relative activity of mouse sera, it was possible to calculate one valid estimate of relative potency for samples A and D and two for sample C. These estimates are tabulated below.

Table 5. — *Results of the Sesardic Method*

Laboratory 6	Sample A	Sample B	Sample C	Sample D
Replicate 1	0.39 (0.27 - 0.59)	-	0.87 (0.61 - 1.31)	-
Replicate 2	-	-	0.72 (0.54 - 0.97)	0.19 (0.12 - 0.30)

(relative potencies and 95 per cent confidence limits)

It appears that the potency estimations of the samples A, C and D calculated on the basis of relative activity of mouse sera are lower than those based on quantal analysis. However the rank order of the potency estimates is consistent with the claimed potencies.

Control antiserum

In general the scores of the control antiserum ranged from 4 to 6. One reason for this variance was that some participants used an initial serum dilution (column 1 of the microtiterplate) of 1:2 and other participants made a dilution series starting with undiluted serum.

Statistical evaluation

In view of the very few valid potency results available for the different types of assay, all valid potencies are taken together in order to compare the Vero cell assay results with the results of both the lethal and intradermal challenge assay results. No attempt is made to determine an intra-laboratory variation.

A one-way analysis of variance was performed on the three groups of data. As far as the samples A, B and C are concerned, no significant difference was found between the potencies calculated by the three types of assay. However for sample B the potencies calculated on basis of the Vero cell assay scores showed a tendency to be higher than the potencies calculated on basis of the challenge data. The *p*-values were 0.599 for sample A, 0.063 for sample B and 0.421 for sample C. The mean potencies and the corresponding values of standard deviation (SD) are tabulated below.

Table 6. — *Mean potencies (IU/ml)*

	Assay method	Number of data	Mean potency (IU per ml)	SD
Sample A	Vero cell assay	17	82.5	48.2
	Intradermal challenge	3	105.4	15.3
	Lethal challenge	3	69.2	28.3
Sample B	Vero cell assay	7	443.1	256.2
	Intradermal challenge	6	191.2	58.8
	Lethal challenge	2	178.9	59.8
Sample C	Vero cell assay	14	89.3	30.4
	Intradermal challenge	4	67.5	32.3
	Lethal challenge	4	83.0	12.3

DISCUSSION

For all three types of assay a rather small number of valid results were obtained. Most laboratories did not succeed in producing valid replicate results, therefore it was not possible to determine variation within laboratories, or to draw conclusions about the significance of differences between laboratories.

For the DT-vaccine, (sample A) and the D-vaccine (sample C) most laboratories succeeded in producing at least one valid estimate of the potency by means of the different types of assay performed. For the DTP-vaccine (sample B) only a few laboratories were able to produce a valid potency estimate by means of the Vero cell assay.

For samples A, B and C no significant difference was found between the potencies determined by either the Vero cell assay, the intradermal challenge assay or the lethal challenge assay.

Only three laboratories were able to assign a potency to the inferior sample D (DT-vaccine) included in the study. For sample D the potencies estimated by Vero cell assay (1.3 IU per ml) and intradermal challenge assay (3.0 IU per ml) correlated well, whereas the potency estimated by lethal challenge assay was significantly higher (32 IU per ml).

The most important reason for the small number of valid assays seems to be that the doses used to immunise the animals were too low. It appears that choosing an immunisation dose merely based on Lf-content is not always convenient. The immunising potency is also dependent on the composition of the vaccine, including the presence of an adjuvant. It is expected that most participants will have fewer invalid assays when dealing with vaccines and reference preparations with which they have become familiar.

It may be concluded from the results that there is no significant difference between the potencies determined by the two Ph. Eur. challenge assays for any of the vaccines tested. In addition, based on the statistically valid Vero cell assays, no significant difference was found between the Vero cell assay and the challenge assays. The mean potencies estimated for sample A (DT-vaccine) and sample C (D vaccine) by Vero cell assay are between the mean potencies estimated by intradermal and lethal challenge assay.

Only a few laboratories were able to estimate a potency for sample B (DTP vaccine) by performing the Vero cell assay. The most important reason for this was the absence of a clear dose-response curve, which appears not to be related to the immunisation dose. In earlier studies [1,4] a lack of homogeneity of assays in mice was found with respect to potency estimates of the tetanus toxoid component of diphtheria-tetanus-pertussis vaccine (adsorbed). In these studies the homogeneity in mice was very much less than that of the assays in guinea-pigs.

(1) Van Ramshorst J.D., Sundarcsan T.K., Outschoorn A.S. (1972). International collaborative studies on potency assays of diphtheria and tetanus toxoids. *Bulletin of the World Health Organization* **46,** 263-276.

In the present study the estimated potencies for the combined diphtheria toxoid (DTP-vaccine) differed in mice and guinea-pigs. The mouse assay gave clearly higher values than the guinea-pig assays. This phenomenon was also found in the earlier studies concerning the tetanus toxoid component of DTP-vaccine. It was ascribed to a possible adjuvant effect of the pertussis component, being more pronounced in mice than in guinea-pigs.

CONCLUSIONS

In view of the small number of valid potency estimates it seems necessary for a laboratory to be familiar with the vaccines tested in the Vero cell assay in order to select immunisation doses resulting in adequate dose-response curves. Provided the appropriate immunisation doses are chosen, the potencies, estimated by Vero cell assay, of the diphtheria vaccine and the combined diphtheria-tetanus vaccine both examined in this study correlate well with the potencies estimated by the Ph. Eur. challenge methods. In all three types of assay the inferior DT-vaccine fails to show any significant immunising potency.

By means of the Vero cell assay it appears to be nearly impossible to assign a potency to the combined diphtheria-tetanus-pertussis vaccine on the basis of this study. In view of similar observations reported by other authors, caution is recommended regarding the implementation of a potency assay using mice as an alternative for assays using guinea-pigs for combined diphtheria-tetanus pertussis vaccines.

2 — Follow-up Study

AIM OF THE STUDY

This study was initiated in order to determine if valid potency estimates can be obtained using the Vero cell assay on the different vaccines included in the collaborative study, provided the appropriate immunisation doses are chosen.

PARTICIPANTS

Both the Laboratory for the Control of Biological Products (LCB) and the Laboratory for Medicines and Medical Devices (LGM) of the RIVM participated in this follow-up study.

MATERIALS AND METHODS

Materials

The Vero cell assay was therefore repeated for samples B (DTP-vaccine) and C (D-vaccine). In this follow-up study again the Ph. Eur working standard (140 IU per ampoule) was included as reference, and the Second International Standard Preparation for Diphtheria toxoid (WHO) was also included (132 IU per ampoule) as well as a commercial batch of DTP-Polio-vaccine, produced by RIVM, acting as an assay-control.

Ph. Eur. working standard

The reference has an assigned potency of 140 IU per ampoule. The initial immunisation dose used in this follow-up study was somewhat higher than the dose used by most participants of the collaborative study (33.33 µl instead of 26.67 µl).

Second International Standard Preparation for Diphtheria toxoid

The standard preparation has an assigned potency of 132 IU per ampoule. The initial immunisation dose used in the present study was 100 µl.

Sample B

The claimed potency of sample B, 140 (100 - 196) IU per ml, is based on the results of one intradermal challenge assay, performed according to the method of the European Pharmacopoeia.

For sample B the initial immunisation dose used was the same as the one used in the collaborative study, i.e. 40 µl.

Sample C

The claimed potency of sample C, 113 (84 - 151) IU per ml, is based on the results of one lethal challenge assay, performed according to the method of the European Pharmacopoeia.

The initial immunisation dose of sample C was more than doubled in the follow-up study compared to the dose used in the collaborative study, i.e. 100 µl instead of 40 µl.

DTP-Polio-vaccine

The claimed potency of the DTP-Polio-vaccine, 254 (174 - 379) IU per ml, is based on the results of one Vero cell assay, performed according to the 'Kreeftenberg method'. The initial immunisation dose of the DTP-Polio-vaccine was 125 µl.

Control antiserum

The control antiserum, used as an assay-control in the collaborative study, was used again by LGM as an assay-control in the follow-up study.

Experimental animals

Male or female N:NIH/RIVM mice specific pathogen free (spf), weighing 10-14 g bred at the RIVM.

Methods

Groups of 40 mice were divided in 5 subgroups of 8 mice. Five two-fold dilutions of the vaccine-samples were prepared in saline. The mice of each subgroup were immunised with 0.5 ml of the vaccine-dilution.

RESULTS

Potencies calibrated against the Ph. Eur. working standard

The results of the Vero cell assay using the Ph. Eur. working standard as reference are tabulated below.

Table 7. — *Results expressed against the Ph. Eur. working standard*

Sample	LCB	LGM
Sample B	no linearity (P = 0.006)	539 (311 - 1166)
Sample C	78 (57 - 112)	78 (53 - 125)*
2nd International Standard	66 (45 - 100) per ampoule	71 (43 - 120) per ampoule
DTP-Polio-vaccine (RIVM)	not tested	88 (58 - 140)

(Estimated Potencies and 95 per cent Confidence Limits: IU per ml).
*no significant parallelism (P = 0.043).

It appears that, applying the appropriate immunisation dose, the potency of sample C can satisfactorily be assayed in the Vero cell assay.

No clear dose-response curve was obtained for sample B by LCB. Though a clear dose-response curve was found by LGM, the potency estimated was much higher than the claimed potency (140 IU per ml). The estimated potency was also much higher than the potencies estimated by the lethal [167 (115 -244) IU per ml; n = 2)] and intradermal challenge assay [191 (151 - 242) IU per ml; n = 4)] as performed in the framework of the collaborative study.

From the Vero cell assay scores it was concluded that this phenomenon cannot be ascribed to an inappropriate range of immunisation doses. It appears that, when calibrated against the Ph. Eur. working standard, the estimated potency of the Second International Standard Preparation is approximately two times lower than the claimed potency (132 IU per ampoule).

Calibrated against the Ph. Eur. working standard the potency of the DTP-Polio vaccine (88 IU per ml) was more than two times lower than the claimed potency (254 IU per ml).

Potencies calibrated against the Second International Standard Preparation

The results of the Vero cell assay of both the laboratories LGM and LCB, using the Second International Standard Preparation as reference, are tabulated below.

Table 8. — *Results expressed against the Second International Standard Preparation*

Sample	LCB	LGM
Sample B	no linearity (P = 0.041) no parallelism (P = 0.031)	1020 (628 - 1840)
Sample C	156 (110 - 226)	155 (106 - 237)*
Ph. Eur. working standard	281 (185 - 413) per ampoule	244 (148 - 394) per ampoule
DTP-Polio-vaccine (RIVM)	not tested	169 (115 ˉ256)

(Estimated Potencies and 95 per cent Confidence Limits: IU per ml).
*no significant parallelism (P = 0.028).

Not surprisingly, the potencies found for all samples tested are approximately two times higher when calibrated against the Second International Standard Preparation instead of the Ph. Eur working standard. The dose-response curve found by LCB for sample B not only significantly deviates from linearity, but also no significant parallelism is found between the dose-response curves of the sample and the Second International Standard Preparation.

Calibrated against the Second International Standard Preparation, the potency of the DTP-Polio-vaccine (169 IU per ml) comes closer to the claimed potency (254 IU per ml).

DISCUSSION

As was expected, the potency of sample C could adequately be estimated by means of the Vero cell assay using an immunisation dose range somewhat higher than the one used in the collaborative study. It appears that the problems encountered by most participants of the collaborative study in producing clear dose-response curves for sample B (DTP-vaccine) is not related to the immunisation dose range, but is likely to be due to an inherent component of the vaccine; in particular, this phenomenon might be ascribed to an adjuvant effect of the pertussis component of the vaccine. However the potency of the DTP-Polio-vaccine produced by RIVM can be estimated well by means of the Vero cell assay, despite the fact that in this vaccine the pertussis component is also present. Therefore it seems that, in addition to the presence of the pertussis component, other properties of the DTP-vaccine (sample B) are responsible for the deviant results of the Vero cell assay.

Rather striking is the difference found between the claimed potencies of both the Ph. Eur. working standard and the Second International Standard Preparation (WHO) and the estimated potencies of these references when calibrated against each other. In practice this will result in estimated potencies of a diphtheria vaccine that are twofold higher if the Second International Standard Preparation is used as a reference instead of the Ph. Eur. working standard (EWS).

It appeared that, when calibrated against the Second International Standard Preparation, the estimated potency of the DTP-Polio-vaccine was much closer to the claimed potency than when calibrated against the Ph. Eur. working standard. This was to be expected in view of the fact that the reference used by RIVM to determine the claimed potency was calibrated against the Second International Standard Preparation, using the Vero cell assay as potency test.

CONCLUSIONS

For the monovalent diphtheria (D) vaccines, as well as the combined diphtheria-tetanus (DT) and diphtheria-tetanus-pertussis-polio (DTP-Polio) vaccines included in the study, the potencies could adequately be calculated on basis of the Vero cell assay results, provided appropriate immunisation doses were used.

It appeared that for one of the combined vaccines included in the study, a diphtheria-tetanus-pertussis (DTP) vaccine, no linear antibody response was found after immunisation of mice with different doses of the vaccine. As a result, for most participating laboratories it was not possible to calculate the potency of this vaccine on basis of the Vero cell assay results.

The results of the follow-up study showed that, compared to the Second International Standard Preparation for Diphtheria toxoid (WHO), the European Working Standard (EWS) is **twice** as potent in the Vero cell assay.

These findings suggest that it is not possible yet for all DTP-vaccines to replace a potency assay based on the protective capacity of a vaccine in guinea-pigs (Ph. Eur. method) by a method based on an immunological response in mice (Vero cell assay). However, the problem that most laboratories encountered in estimating potency of the DTP-vaccine by means of the Vero cell assay may be dependent on the strain of mice used.

In earlier studies a lack of homogeneity of the immunological response of mice to tetanus toxoid component of DTP-vaccine was found. This phenomenon may be inherent to the composition of the vaccine and due to an adjuvant effect of the pertussis component of the vaccine, in combination with the adsorbent used. Authors of earlier studies suggested that this adjuvant effect may be more pronounced in mice than in guinea-pigs.

However, this does not mean the estimation of diphtheria toxoid potency in pertussis toxoid-containing vaccines is always impossible using an assay based on the immunological response of mice. For example, the Vero cell assay of combined DTP-Polio vaccine, produced by the RIVM, was successfully validated against the Ph. Eur. lethal challenge test in guinea-pigs.

Another item to be discussed is the reference preparation for diphtheria vaccines to be used in the Vero cell assay. The potency assigned to the Second International Standard Preparation (132 IU per ampoule) is based on the results of both challenge tests and antibody titration tests in guinea-pigs, using the First International Standard Preparation as reference.

The potency assigned to the Ph. Eur. working standard (140 IU per ampoule) is based on the results of a combination of lethal and intradermal challenge assays in guinea-pigs, using the First International Standard Preparation as reference. However, the estimated potencies of the Ph. Eur. working standard and the Second International Standard Preparation, when calibrated against each other in the Vero cell assay, significantly differ from the potencies officially assigned to these standard preparations.

Therefore it seems that the relationship between the Ph. Eur. working standard and the Second International Standard Preparation, as it was found for assays in guinea pigs, does not guarantee the same relationship in the Vero cell assay.

It is recommended that the Vero cell assay be validated for each formulation of diphtheria vaccine separately. No significant deviation from linearity, nor parallelism should be found. The strain of mice used in the Vero cell assay deserves particular attention, for some strains may be more appropriate than others.

Furthermore it is recommended that the exact relationship between the potency of the Ph. Eur. working standard and the Second International Standard Preparation for Diphtheria toxoid, as they are estimated by the Vero cell assay should be determined in a small-scale collaborative study.

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LIST OF PARTICIPANTS

I.S. AABERGE National Institute of Public Health, Department of Vaccines, Oslo, Norway.

H. AGGERBECK Statens Scruminstitut, Bacterial Vaccine Department, Copenhagen, Denmark.

R. DOBBELAER Instituut voor Hygiene en Epidemiologie, Brussels, Belgium.

M. DUCHENE SmithKline Beecham Biologicals, Rixensart, Belgium.

J. GIROUX Laboratoire National de la Santé, Montpellier, France.

M. GOMMER Laboratory for Medicines & Medical Devices LGM, Bilthoven, Netherlands.

A. HACKER-RIEDER Abteilung Mikrobiologie, Bundesstaatliches Serumprüfungsinstitut, Vienna, Austria.

H. HARTMAN FRIES & A-M.VANGSTED National Board of Health, Medicines Division, Bronshoj, Denmark.

U. KLOCKMANN Behringwerke AG, Marburg, Germany.

R.M. OLANDER National Public Health Institute, Vaccine Laboratory, Helsinki, Finland.

L. PEYRON Pasteur Mérieux Sérums et Vaccins, Marcy l'Etoile, France.

S. RICO Laboratorio de Bacteriologia Instituto de Salud Carlos III, Madrid, Spain.

D. SESARDIC Division of Bacteriology, NIBSC, Potters Bar, UK.

J. STALDER Federal Office of Public Health, Section for Immunobiological Prod., Bern, Switzerland.

P. TROMMELMANS Service de Contrôle de Médicaments, Association Pharmaceutique Belge, Brussels, Belgium.

J. W. van der GUN Laboratory for Control of Biological Products, Bilthoven, Netherlands.

S.J. VARALLYAY Swiss Serum and Vaccine Institute, Biological Quality Control, Bern, Switzerland.