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Collaborative Study: Standardization of Japanese Reference Diphtheria and Tetanus Toxoids, (Adsorbed, Lot 2), for Potency Determination of Diphtheria-Tetanus-Acellular Pertussis Combined Vaccine

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The potency of toxoid products is measured directly by the protection of guinea pigs or mice from the killing effect of toxins or by measuring in vivo or in vitro the neutralizing activity of the sera from immunized animals (1). Here, the common principle is to compare the potency of test products

with that of the standard/reference toxoid.

In measuring the potency of combined diphtheria-tetanus-pertussis (DTP) vaccines, the suitable standards/references are combined toxoids, not single diphtheria or tetanus toxoids (2-4). However, the international standards are available only for single toxoids and not for the combined ones. This is mainly because the potency of diphtheria or tetanus toxoids, in humans and in experimental animals, is greatly affected by the third component, pertussis vaccine.

Japanese reference material for the quality control of diphtheria and tetanus toxoids for DTP vaccines (lot 1) was first produced in 1985 as adsorbed diphtheria-tetanus-acellular pertussis combined vaccine (DTaP). As lot 1 was nearly used up 13 years after its production, a new lot, lot 2, was established in 1998 by calibration against the Japanese reference product lot 1. The calibration experiments were conducted as a collaboration among of Chemo-Sero-Therapeutic Research Institute (Kumamoto), Chiba Serum Institute (Chiba), Denka Seiken Co. (Niigata), the Kitasato Institute (Saitama), the Research Foundation for Microbial Diseases of Osaka University (Kagawa), and Takeda Chemical Industries, Ltd. (Yamaguchi). All the participants used the Japanese minimum requirement for quality control of biologicals (5) as the standard.

The candidate adsorbed DTaP vaccine was prepared in the Hikari plant of Takeda Chemical Industries, Ltd. It contained 47 Lf/vial of diphtheria toxoid, 2.5 Lf/vial of tetanus toxoid, 15 µg pN/vial of acellular pertussis vaccine, 0.2 mg/vial of adjuvant aluminium hydroxide, and 17.5 mg/vial of Hemacel as a stabilizer. Guinea pigs (female, Hartley strain, 300-400 g, "clean" grade, 10 animals/group) and mice (female, ddY strain, 5 weeks old, SPF grade, 10 animals/group) were injected subcutaneously with the vaccines at doses of 2 ml and 0.5 ml, respectively. For diphtheria toxoid potency tests using guinea pigs, the vaccines were diluted in physiological saline solution at 20-, 48-, 115-, and 277-fold for the reference lot 1 and at 40-, 96-, 230-, and 554-fold for the candidate. For mice assays, dilutions were at 4-, 8-, 16-, and 32-fold for reference lot 1 and at 8-, 16-, 32-, and 64-fold for the candidate. For tetanus toxoid potency tests using guinea pigs, the vaccines were diluted at 20-, 48-, 115-, and 277-fold, and those for mice at 25-, 50-, 100-, and 200-fold for both lot 1 and the candidate.

Four weeks after immunization, guinea pigs and mice were

partially bled, and any animals that died as a result of bleeding were discarded. Four days after bleeding, animals were challenged with diphtheria test toxin lot K-1 or tetanus test toxin lot TA-4B (50 times LD₅₀/guinea pig, 100 times LD₅₀/mouse), and symptoms were observed for 7 days.

Sera from the immunized guinea pigs and mice were subjected to diphtheria antitoxin potency assay in the VERO cell culture (6,7) using diphtheria toxin for culture use, lot M59, and the national standard diphtheria antitoxin lot 9 as a reference antitoxin. Sera from immunized guinea pigs were subjected to anti-diphtheria and anti-tetanus potency assays by using the KPA kit (polyaminoacid particle-based passive agglutination test produced by the Chemo-Sero-Therapeutic Research Institute).

Observed symptoms developing in challenged animals were converted to scores, essentially as described by Watarai et al. (8) for the diphtheria potency test and by Murata (9,10) for the tetanus potency test. All the results (including the Vero and KPA kit assays) were submitted to statistical analysis by the parallel line assay method, using a statistical software package designed for the quantitative quality control of biologicals, prepared according to Finney (11).

Guinea pig toxin challenge assays gave potencies of 64 units/ml for diphtheria toxoid and 40 units/ml for tetanus toxoid (Table 1). These values satisfy the criteria of the Japanese minimum requirement for quality control of biologicals. Under authorization by the Ministry of Health, Labour and Welfare of Japan, they are now distributed by the National Institute of Infectious Diseases (NIID, Tokyo) respectively as "National reference preparation of diphtheria toxoid, adsorbed for DPT combined vaccine, Lot 2" and "National reference preparation of tetanus toxoid, adsorbed for DPT combined vaccine, Lot 2".

Tables 1 and 2 respectively summarize statistical analyses of the guinea pig challenge studies for diphtheria and tetanus toxoids. Assays were conducted three times at NIID and once at the other laboratories, A to F. The summary data is shown in Table 3. The data obtained by the different methods were mutually consistent. However, for diphtheria toxoid, the mouse cell culture assay tended to give values slightly higher than the guinea pig challenge assay. Meanwhile, for tetanus toxoid, the guinea pig assay tended to give values slightly higher than did the mouse assay. Though the difference was statistically insignificant, the observation should be taken into

Table 1. Potency of diphtheria toxoid measured by guinea pig challenge method: statistical analysis

Site of measurement		Variance			Regression coefficient			Potency (units)	
		lot 1	Candidate	Common	lot 1	Candidate	Common	Mean	Confidence interval
NIID	exp 1	0.978	1.14	1.03	3.92	3.70	3.82	71.8	48.1 - 102
	exp 2	0.933	3.3	1.05	1.58	3.11	2.93	104	38.9 - 195
	exp 3	3.25	2.10	2.61	2.21	3.14	2.66	66.2	31.6 - 119
A		1.17	4.26	3.23	3.00	2.87	2.94	102	47.4 - 197
B		1.09	1.33	1.21	2.65	2.93	2.79	39.2	22.3 - 61.2
C		2.82	2.49	2.71	3.73	3.88	3.81	79.1	47.9 - 123
D		0.857	1.82	1.72	3.08	3.08	3.08	33.0	15.9 - 56.3
E		1.14	2.22	1.27	2.74	3.04	2.88	66.9	36.5 - 104
F		0.986	1.66	1.29	2.03	2.68	2.35	36.4	16.8 - 63.6
Mean of 9 experiments		1.56	2.03	1.86	2.71	3.22	3.11	64.4	54.7 - 75.7

Experiments were repeated three times at the NIID and once at other sites (indicated as A to F). Variance and correlation coefficients were calculated from scores of lot 1-immunized animals and candidate-immunized animals, separately and combined (indicated as "common"), at each site. Potency was calculated as described in the text. The geometrical mean of the results and a confidence interval at *P* = 0.95 are shown.

Table 2. Potency of tetanus toxoid measured by guinea pig challenge method: statistical analysis

Site of measurement	Variance			Regression coefficient			Potency (units)	
	lot 1	Candidate	Common	lot 1	Candidate	Common	Mean	Confidence interval
NIID exp 1	0.318	0.647	0.431	2.36	3.07	2.88	51.8	39.6 - 67.7
exp 2	0.786	0.687	1.01	4.04	4.78	4.39	30.7	22.7 - 41.5
exp 3	1.50	0.214	0.739	3.42	3.47	3.44	65.2	49.5 - 85.9
A	1.16	0.600	0.822	5.73	3.54	3.98	27.5	20.0 - 38.0
B	2.02	0.281	1.09	2.38	3.38	2.88	36.2	24.3 - 54.0
C	0.444	1.67	1.12	4.81	4.64	4.72	33.6	25.5 - 44.4
D	1.15	1.44	0.838	4.18	6.45	4.61	38.1	28.7 - 50.5
E	0.665	0.518	0.579	3.99	3.15	3.39	40.1	30.9 - 51.8
F	0.778	0.712	0.747	4.06	3.52	3.78	52.7	39.9 - 69.4
Mean of 9 experiments	0.898	0.515	0.725	4.01	3.41	3.64	40.4	36.3 - 44.9

See footnote of Table 1.

Table 3. Summary of potency measurement

Animal	Assay method	Potency (units)			
		Diphtheria		Tetanus	
		Geometrical mean	Confidence interval	Geometrical mean	Confidence interval
Guinea pig	Toxin challenge	64.4	54.7 - 75.7	40.4	36.3 - 44.9
	VERO cell culture	78.1	67.3 - 90.6	ND ¹⁾	ND
	KPA	56.2	47.8 - 66.2	40.6	36.7 - 44.9
Mouse	VERO cell culture	76.8	65.3 - 90.3	ND	ND
	Toxin challenge	ND	ND	34.4	30.8 - 38.6

Experiments were repeated nine times (three times at the NIID and once at each of six manufacturers described in the text). The geometrical mean of results and confidence interval at $P = 0.95$ are shown.

¹⁾: ND, not determined.

account when these lots are used as reference materials. The guinea pig and mouse cell culture assays gave almost identical potency values.

Current routine potency assays in Japan are mouse assays using VERO cell culture for diphtheria and toxin challenge assay for tetanus. In such assays, in order to avoid under- or overestimation, the above-indicated slight differences brought about by different methods should be taken into account. Our joint research group produced 1,910 vials of lot 2 (955 for use as diphtheria reference and 955 for use as tetanus reference), which will satisfy the demand in Japan for more than 10 years.

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