Influence of Temperature on the Efficacy of 2-Phenoxyethanol as a Preservative for Adsorbed Diphtheria-Purified Pertussis-Tetanus Combined Vaccine

Eiji Komatsu,[a] Hiroyuki Yamazaki,[a] Katsuyo Abe,[a] Shigeharu Iyama,[a] Mitsuo Oishi,[a] Takaaki Sato,[a] Chiharu Yoshino,[b] Hiroshi Hashimoto,[b] Mineo Watanabe,[c] and Masaaki Nagai*,[a]

[a]Division of Bacterial Vaccines and [b]Division of Quality Control, Research Center for Biologicals, The Kitasato Institute, 6–111 Arai, Kitamoto, Saitama 364–0026, Japan and [c]Laboratory of Pertussis, Department of Microbiology and Biologics, Daiichi College of Pharmaceutical Sciences, 22–1 Tamagawa-cho, Minami-ku, Fukuoka 815–8511, Japan

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The efficacy of two antimicrobial preservatives, 2-phenoxyethanol (2-PE) and Thimerosal, was compared in adsorbed diphtheria-purified pertussis-tetanus combined vaccine (DPT vaccine). Thimerosal had strong microbicidal activity against Gram-positive bacteria, Gram-negative bacteria, yeast and fungi at 25°C and 4°C. 2-PE also had microbicidal activity against these microorganisms, only had microbistatic activity against fungi at 4°C. Neither 2-PE nor Thimerosal affected the potency or toxicity of the DPT vaccine. These data suggest that 2-PE has weaker antimicrobial activity than Thimerosal against yeast and fungi in DPT vaccine at low temperatures.

Key words —— DPT vaccine, 2-phenoxyethanol, Thimerosal, preservative

INTRODUCTION

Inactivated bacterial vaccines, such as adsorbed diphtheria-purified pertussis-tetanus combined vaccine (DPT vaccine), are commonly packaged in multi-dose vials.[1,2] Preparations usually include an antimicrobial preservative to prevent the growth of contaminants that might be introduced during withdrawal of a dose of vaccine from such vials. Thimerosal is commonly used at a concentration of 0.01% (w/v) as such a preservative. It is a very effective preservative containing mercury, and has been used in various vaccines since the 1930s. However, the use of mercury-containing compounds in vaccines is considered to be potentially harmful, and it has also been found that some components of vaccine are labile in the presence of Thimerosal.[3] Therefore, other preservatives such as 2-phenoxyethanol (2-PE) are being evaluated for use in preparations of inactivated polio-DPT combined vaccine.[4] Lowe and Southern reported that the antimicrobial activities of Thimerosal and 2-PE were similar in a combination of diphtheria toxoid, tetanus toxoid and whole-cell pertussis vaccine.[5] However, to our knowledge there have been no reports in which the antimicrobial effectiveness of Thimerosal and 2-PE in purified DPT vaccines has been compared. In this study, we examined the antimicrobial activities in DPT vaccine of these two preservatives, as well as their effects on the potency and toxicity of this vaccine.

MATERIALS AND METHODS

DPT Vaccine —— The DPT vaccine, a co-purified vaccine, was manufactured by the Kitasato Institute, Tokyo, Japan.[6] The vaccine contained formalin-treated pertussis components (pertussis toxin, filamentous hemagglutinin, pertactin and fimbriae), diphtheria toxoid and tetanus toxoid. The concentration of aluminum salt was approximately 0.17 mg/ml. Thimerosal or 2-PE (Wako Pure Chemical Industries, Osaka, Japan) was added as a preservative to aliquots of the vaccine at a final concentration of 0.01% (w/v) or 0.5% (w/v), respectively. The vaccine was then transferred aseptically to sterile 10-ml vials, which were sealed with sterile multi-puncture rubber stoppers that were held in place with a crimped aluminum overseal.

Biological Tests of the DPT Vaccine —— Tests of the potency and toxicity of the preparations of the vaccine were performed in accordance with the Japanese minimum requirements for biological products.[1] The potency of the pertussis vaccine was assayed using a mouse intracerebral challenge test. Potency of the diphtheria toxoid was determined in mouse by the antitoxin titration method, and that of the tetanus toxoid was measured in mouse by the toxin challenge method. The abnormal toxicity tests

*To whom correspondence should be address: Division of Bacterial Vaccines, Research Center for Biologicals, The Kitasato Institute, 6–111 Arai, Kitamoto, Saitama 364–0026, Japan. Tel. & Fax: +81-48-593-3852; E-mail: nagai-m@kitasato.or.jp.

were performed using guinea pigs and mice. Body weight-decreasing units (BWDU) were determined by the decrease in body weight of mice after 16 hr. The lymphocytosis-promoting units (LPU) were determined by the leukocyte increase in peripheral blood of mice after 3 days, and histamine-sensitizing units (HSU) were measured by rectum temperature method in mice.

Preservative-Effectiveness Tests —— These tests measured the antimicrobial activity, as described in the general information of the 13th edition of the Japanese Pharmacopoeia (JP13).[7] Microorganisms were obtained from the Japan Collection of Microorganisms (Wako, Japan) and from the Institute for Fermentation (Osaka, Japan). The microorganisms tested and the respective culture conditions are shown in Table 1. The culture conditions were those recommended in JP13 in all cases. The bacterial and yeast cells that had been grown in liquid medium were collected by centrifugation for 10 min at $4500 \times g$. Each pellet was washed by resuspension in sterile phosphate-buffered saline (PBS). Then the suspension in PBS was centrifuged and cells were resuspended in sufficient sterile PBS to yield a microbial count of approximately 1×10^8 colony-forming units (CFU)/ml. Fungal cells on agar-solidified medium were harvested in sterile PBS that contained 0.05% (w/v) polysorbate 80, and sufficient sterile PBS was added to yield a cell count of approximately 1×10^8 CFU/ml. Volumes of 10 ml of vaccine were challenged with 0.1 ml of suspended cells (1×10^6 CFU/ml). Ten vials were tested with each microorganism, and vials were incubated in sets of five vials at 4°C and 25°C. One milliliter of each sample was withdrawn after 7, 14, 21 and 28 days. The number of CFU of microorganisms was determined in each sample of vaccine by the pour plate method, as described in the microbial limit tests in JP13.[8]

RESULTS AND DISCUSSION

The effects of 2-PE on the toxicity and potency of the DPT vaccine are shown in Table 2. The mouse toxicity (body weight-decrease, leukocyte-increase

Table 1. Microbial Strains and Culture Conditions

Species	Strain	Culture medium for		Incubation temperature (°C)
		inoculation	colony count	
Escherichia coli	JCM1649	SCD[a] broth	MacConkey agar	37
Pseudomonas aeruginosa	IFO13275	SCD broth	SCD agar	37
Staphylococcus aureus	JCM2151	SCD broth	Mannitol-salt agar	37
Candida albicans	JCM2085	SCD broth	PDA[b]	25
Aspergillus niger	IFO9455	PDA	PDA	25

a) Soybean-casein digest. *b*) Potato dextrose agar.

Table 2. Effects of 2-PE on the Toxicity and Potency of the DPT Vaccine

Tests	Preservatives (mean ± S.D.)		Release specifications
	2-PE[a]	Thimerosal[b]	
Toxicity			
Abnormal toxicity	No toxicity	No toxicity	No toxicity
Mouse body weight-decreasing toxicity	7.8 ± 4.88	5.6 ± 1.98	≤ 10 BWDU[c]/ml
Mouse leukocyte-increasing toxicity	0.191 ± 0.060	0.15 ± 0.087	≤ 0.5 LPU[d]/ml
Mouse histamine-sensitizing toxicity (4°C)[e]	0.11 ± 0.024	0.094 ± 0.061	≤ 0.4 HSU[f]/ml
Mouse histamine-sensitizing toxicity (37°C)[g]	0.115 ± 0.028	0.098 ± 0.057	≤ 0.4 HSU/ml
Diphtheria toxin activity	Detoxified	Detoxified	Detoxified
Tetanus toxin activity	Detoxified	Detoxified	Detoxified
Potency			
For pertussis vaccine	18 ± 4.23	25 ± 11.3	≥ 8 U/ml
For diphtheria toxoid	80 ± 12.27	72 ± 18.7	≥ 47 IU/ml
For tetanus toxoid	96 ± 21.57	62 ± 19.0	≥ 27 IU/ml

a) The data are averages of results of three independent examinations. *b*) The data are averages of results of 15 independent examinations. *c*) Body weight-decreasing units. *d*) Lymphocytosis-promoting units. *e*) Incubation at 4°C for 4 weeks. *f*) Histamine-sensitizing units. *g*) Incubation at 37°C for 4 weeks.

Table 3. Antimicrobial Effects of 2-PE and Thimerosal in DPT Vaccine

Incubation temperature	Preservative	Microorganism	% of inoculum count after (mean ± S.D.)				Judgment[a)]
			7 days	14 days	21 days	28 days	
25°C	2-PE	*E. coli*	N.D.[b)]	N.D.	N.D.	N.D.	Conform
		P. aeruginosa	N.D.	N.D.	N.D.	N.D.	Conform
		S. aureus	N.D.	N.D.	N.D.	N.D.	Conform
		C. albicans	< 0.1	< 0.1	N.D.	N.D.	Conform
		A. niger	7.4 ± 2.4	0.7 ± 0.25	< 0.1	< 0.1	Conform
	Thimerosal	*E. coli*	N.D.	N.D.	N.D.	N.D.	Conform
		P. aeruginosa	N.D.	N.D.	N.D.	N.D.	Conform
		S. aureus	N.D.	N.D.	N.D.	N.D.	Conform
		C. albicans	N.D.	N.D.	N.D.	N.D.	Conform
		A. niger	N.D.	N.D.	N.D.	N.D.	Conform
	None	*E. coli*	< 0.1	< 0.1	N.D.	N.D.	Conform
		P. aeruginosa	0.4 ± 0.03	< 0.1	< 0.1	< 0.1	Conform
		S. aureus	1 ± 0.5	< 0.1	N.D.	N.D.	Conform
		C. albicans	157 ± 16.1	124 ± 16.8	42 ± 8.8	90 ± 16.1	Not conform
		A. niger	88 ± 22.0	52 ± 27.7	52 ± 9.1	56 ± 26.0	Not conform
4°C	2-PE	*E. coli*	< 0.1	N.D.	N.D.	N.D.	—[c)]
		P. aeruginosa	N.D.	N.D.	N.D.	N.D.	—
		S. aureus	448 ± 55.2	0.9 ± 0.42	N.D.	N.D.	—
		C. albicans	612 ± 73.9	29 ± 5.1	5.3 ± 2.9	0.2 ± 0.19	—
		A. niger	64 ± 39.2	50 ± 22.5	60 ± 16.2	62 ± 9.4	—
	Thimerosal	*E. coli*	N.D.	N.D.	N.D.	N.D.	—
		P. aeruginosa	N.D.	N.D.	N.D.	N.D.	—
		S. aureus	N.D.	N.D.	N.D.	N.D.	—
		C. albicans	N.D.	N.D.	N.D.	N.D.	—
		A. niger	N.D.	N.D.	N.D.	N.D.	—
	None	*E. coli*	6 ± 1.0	< 0.1	< 0.1	N.D.	—
		P. aeruginosa	43 ± 2.3	21 ± 3.3	5 ± 1.7	1.4 ± 0.38	—
		S. aureus	210 ± 64.8	180 ± 29.2	178 ± 13.2	61 ± 19.6	—
		C. albicans	158 ± 36.5	71 ± 12.3	58 ± 9.1	55 ± 5.3	—
		A. niger	70 ± 17.5	42 ± 9.6	49 ± 19.2	45 ± 20.0	—

The data are averages of results of three independent examinations. *a*) Bacteria: Reduction of more than 0.1% of the viable bacterial count in mixed samples within 14 days of the initial inoculum and the viable cell count remaining at the same level or decreasing at 28 days after challenge. Yeast and fungi: The viable count of yeast and fungi in mixed samples at the same level or less than the inoculum at 14 and 28 days after inoculation. *b*) N.D.: Not detected (less than 0.0001%). *c*) —: There is no criterion for this test at 4°C.

and histamine-sensitizing toxicity) levels of the samples of vaccine that contained 2-PE were similar to those of the samples that contained Thimerosal. The potency of the DPT vaccine with respect to pertussis, diphtheria and tetanus was higher than the release specifications of the vaccine. These data suggest that neither 2-PE nor Thimerosal affected the potency or toxicity of the vaccine.

The antimicrobial activities of the two preservatives in the vaccine are shown in Table 3. No viable microorganisms were recovered from any samples of vaccine containing Thimerosal after incubation at 4°C or 25°C, when samples were examined 7, 14, 21 and 28 days after the start of incubation. These data indicate that Thimerosal at 0.01% had strong microbicidal activity against Gram-positive bacteria, Gram-negative bacteria, yeast and fungi at these temperatures. The microbicidal activity of 2-PE against these microorganisms was apparent at 25°C, but the activity against yeast and fungi was weaker than that of Thimerosal; however, both substances passed the preservative-effectiveness tests of JP13.[7] 2-PE exhibited only microbistatic activity against fungi at 4°C (Table 3). The action of 2-PE on Gram-negative bacteria was reported to involve the disruption of cell membranes and the uncoupling of oxidative phosphorylation.[9–12] It might have similar effects on Gram-positive bacteria, yeast and fungi,

however, the effects might be dependent on incubation temperature in these cases. Since DPT vaccines are usually stored at temperatures between 2, and 10°C, 2-PE has a disadvantage in terms of its antimicrobial activity at low temperatures.

In conclusion, our results could be taken to indicate that 2-PE is suitable for use in DPT vaccines. However, 2-PE had lower antimicrobial activity than Thimerosal against yeast and fungi in our vaccine at low temperature. Therefore, 2-PE should be used carefully, with aseptic precautions, if it is to be used as a preservative in DPT vaccines. Further studies are now required on the long-term stability of DPT vaccines that contain 2-PE.

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