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Evaluation of endotoxin content of diphtheria–tetanus-acellular pertussis combined (DTaP) vaccines that interfere with the bacterial endotoxin test

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Abstract

Applicability of the endotoxin test to diphtheria–tetanus-acellular pertussis combined (DTaP) vaccines was examined. We found some DTaP vaccines that strongly interfered with Limulus amoebocyte lysate (LAL) activity of endotoxin without affecting lethal activity of endotoxin in D-galactosamine-treated mice. LAL activity that was interfered in such vaccines increased apparently after the treatment with phosphate buffer at 4 °C for a week. The DTaP vaccines that interfered with the endotoxin test showed no significant effect on endotoxin activity in inducing tumor necrosis factor-alpha (TNF-α) in rabbit peripheral blood. The in vitro TNF-α induction assay was, therefore, suggested to be an appropriate assay method for the quantitative detection of the endotoxin activity in DTaP vaccines.

Keywords: Endotoxin; Pyrogen; DTP vaccine

1. Introduction

Lipopolysaccharide (LPS) of gram-negative bacteria, which is also referred to as endotoxin, is known to have various biological activities [1]. Even a very small amount of it causes fever in human [2]. Contamination of parenteral drugs with endotoxin, therefore, has been a serious threat. Contamination of endotoxin in diphtheria–tetanus-acellular pertussis combined (DTaP) vaccine, which is formally referred to as precipitated diphtheria–tetanus-purified pertussis combined vaccine, has been regulated by the mouse body weight decreasing (BWD) toxicity test in the Minimum Requirements for Biological Products of Japan [3]. Although the BWD toxicity test has played a key role to control pyrogenic activity of DTaP vaccines [4], the test is much less sensitive than the Limulus amoebocyte lysate (LAL) test. Thus, it seems preferable to replace the current BWD toxicity test by a more sensitive test for the regulation of endotoxin in DTaP vaccines.

The bacterial endotoxin test (the LAL test), which was first developed by Levin and Bang [5], is based on highly sensitive clotting of LAL in the presence of endotoxin. Methods and reagents for the LAL test have been remarkably improved [6,7], thereafter, to allow its wide application [3,8–10]. The World Health Organization (WHO) issued its guidelines for acellular pertussis vaccines in 1998 to state that residual endotoxin content should be tested by means of the LAL test or other appropriate assay [11]. However, we found some DTaP vaccine batches that strongly interfere with LAL activity of endotoxin without affecting its in vivo activities such as pyrogenicity in rabbits [12] and lethal activity in D-galactosamine-treated mice. For the application of the LAL test to the regulation of endotoxin in such DTaP vaccine batches, it is required to find a way to eliminate the interfering effect of the vaccine preparations. Here we report the method for eliminating the interfering effect of the DTaP vaccines on the LAL test. We also propose the use of tumor necrosis factor-alpha (TNF-α) induction in peripheral blood of rabbits [13] for detection of endotoxin in DTaP vaccines.

2. Materials and methods

2.1. Commercial DTaP vaccines

The DTaP vaccine batches subjected to the national quality control tests during 1997 and 1999 in Japan were used in the present study. The vaccines were comprised of detoxified pertussis antigens, mainly pertussis toxin (PT)

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and filamentous haemagglutinin (FHA), and diphtheria and tetanus toxoids adsorbed to about 0.16% Al/ml of aluminum hydroxide gel (Al-hydro gel) as adjuvant.

2.2. Lipopoplysaccharide

Lipopolysaccharide (Bp-LPS) was extracted and purified from *Bordetella pertussis* phase I Tohama strain by Westphal's phenol water method [14]. It was used to perform the inhibition/enhancement test. The Bp-LPS was suspended at a concentration of 1.0 mg/ml with pyrogen-free distilled water and kept at 4 °C without detectable change in LAL activity. LAL activity of Bp-LPS was measured in parallel with Japanese national reference standard endotoxin (lot 3, 13,000 endotoxin units (EU)/vial) [15] and endotoxin units of Bp-LPS were estimated to be 8000 EU/μg.

2.3. The bacterial endotoxin test

The Bp-LPS and DTaP vaccines were serially diluted with pyrogen-free distilled water at four-fold intervals. A 50 μl-volume each of the dilutions was mixed with an equal volume of the LAL reagent (Endospecy; Seikagaku Corp., Tokyo, Japan) of which reactivity to 1,3-β-D-glucan is removed [16]. Rate of color development was measured using a microplate reader (WellReader SK601; Seikagaku Corp.). Endotoxin content of samples was calculated according to the parallel line assay method [17] using logarithmically transformed concentrations and the rates of color development. The values were expressed as EU or μg using Bp-LPS of known EU value and concentration as a reference.

2.4. Mouse lethal activity test

Female mice of 10 weeks old C3H/HeN of LPS-responder strain (SPF grade; Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used. Random groups of five mice were housed in each cage and kept at about 23 °C in an animal room. Lethal activity of a test sample was measured using D-galactosamine-sensitized mice [18]. Groups of 10 mice each were intravenously injected with a 0.2-ml volume of a mixture of sample preparations and 20 mg per mouse of D-galactosamine hydrochloride (Sigma Chemical Co., MO, USA) dissolved in pyrogen-free physiological saline. Deaths of the mice were recorded up to 24 h after the injection.

2.5. In vitro TNF-α induction assay

The test was carried out as previously reported [13]. Briefly, fresh blood was collected from female rabbits each of Japanese white strain (Kitayama Labes Co. Ltd., Nagano, Japan or Japan Laboratory Animals Inc., Tokyo, Japan). An aliquot of 0.1 ml of appropriately diluted samples with pyrogen-free physiological saline was gently mixed with 0.15 ml of heparinized rabbit blood and 0.75 ml of pyrogen-free physiological saline in a pyrogen-free centrifuge tube. The mixture in duplicate was incubated at 37 °C for 5 h. Supernatants were isolated by a centrifuge at 500 × *g* for 2 min and stored frozen at −20 °C. The thawed supernatants were appropriately diluted with 25% (v/v) Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) in distilled water and subjected to the measurement of TNF-α concentration by an enzyme-linked immunosorbent assay (ELISA) using anti-rabbit TNF-α goat immunoglobulin G (IgG), biotin labeled anti-rabbit TNF-α goat IgG (Research Diagnostics Inc., NJ, USA) and horseradish peroxidase-conjugated streptavidin (Chemicon International Inc., CA, USA). TNF-α concentrations in the supernatants were calculated according to the parallel line assay method with rabbit TNF-α preparation (Research Diagnostics Inc.) as a reference using logarithmically transformed concentrations and absorbance values.

3. Results

3.1. Application of the LAL test

We reported that the interfering effect of DTaP vaccines on LAL activity of added Bp-LPS progressed gradually at 4 °C [12]. We, therefore, examined the effect on the lethal activity of Bp-LPS in D-galactosamine-treated mice using DTaP vaccine batch A, that interfered with the LAL activity (Table 1).

All test samples were prepared simultaneously and kept at 4 °C. Two weeks later, the LAL activity and the lethal activity of the samples were measured. When LPS concentrations in the vaccine preparations were compared with the control Bp-LPS samples diluted in saline by the LAL test, it was estimated that the concentration in the vaccine preparation added with 0.002 μg per mouse of Bp-LPS was

Table 1
Effect of DTaP vaccines on the lethal activity of LPS in D-galactosamine-treated mice

Samples	Added LPS (μg per mouse)	LAL test (μg per mouse)	Lethal activity[a]	
			Dead/total	Rate (%)
Saline	0	Negative	0/10	0
DTaP batch A	0	Negative	0/10	0
Saline + LPS[b]	0.00067	0.00067	0/10	0[c]
	0.001	0.001	1/10	10
	0.002	0.002	12/30	40
	0.01	0.01	16/20	80
DTaP batch A + LPS[b]	0.002	0.00067	5/10	50[c]

[a] Lethal activity during 24 h after intravenous injecting 10-week-old female mice of C3H/HeN strain with a mixture of 20 mg per mouse of D-galactosamine and a sample.
[b] Kept at 4 °C for 2 weeks before measurements.
[c] Significantly deferent at $P < 0.05$.

0.00067 μg per mouse corresponding to 33.3% of the activity (Table 1). On the other hand, the Bp-LPS in the vaccine preparation retained the similar level of lethal activity to that of 0.002 mg per mouse of the control Bp-LPS that killed 40% of D-galactosamine-treated mice.

DTaP vaccine batch A and the control Bp-LPS in distilled water were added with sodium citrate, a chelating agent, at a final concentration of 1.0% and kept at 4 °C for 48 h to examine the possibility of eliminating the interfering effect of Al-hydro gel on the LAL activity because we reported previously that Al-hydro gel alone (0.16% Al/ml) caused a remarkable reduction in LAL activity of added Bp-LPS [12]. The LAL activity of the vaccine preparation was estimated in reference to the control Bp-LPS containing the same concentration as Bp-LPS added to the vaccine. Recovery of the LAL activity of Bp-LPS added to the vaccine preparation was only 21.6% after the treatment. While DTaP vaccine batches A and B were treated with phosphate buffer (pH 8.0) at a final concentration of 0.25 M at 4 °C, LAL activity of Bp-LPS added to the vaccine preparations increased gradually. A week later it came to the similar level of LAL activity to the control Bp-LPS that had been subjected to the same treatment (Fig. 1). Only about 50% of the LAL activity was recovered by that time when Bp-LPS had been added to Al-hydro gel alone. It took 3 weeks for the Bp-LPS added to Al-hydro gel to recover its original level of LAL activity by the treatment (data not shown).

3.2. Application of the in vitro TNF-α induction assay

Although the treatment with phosphate buffer was shown to be effective in eliminating the interfering effect of DTaP vaccines on LAL activity, it took a rather long time for the complete recovery of the activity. We evaluated the in vitro TNF-α induction assay using peripheral blood of rabbits as an alternative for testing endotoxin content of DTaP vaccines.

We examined the possibility of dose-dependent interference by DTaP vaccine with the TNF-α production due to cytotoxicity on the blood cells. The assay was carried out immediately after adding Bp-LPS to serial dilutions of DTaP vaccines so as to avoid the influence of the effect that causes reduction of LAL activity. Two batches of DTaP vaccine, batch A that interfered with LAL activity and batch C that did not, were used. The vaccines were serially diluted from 1/1 to 1/8 at two-fold intervals and added with Bp-LPS at a final concentration of 5.0 EU/ml. Dilutions of Bp-LPS in saline were prepared as a reference. The measurement of the vaccine preparations and the Bp-LPS dilutions was repeated three times using the blood from each of three rabbits. TNF-α induction activity of Bp-LPS in the vaccine preparations was calculated in reference to that of the Bp-LPS dilutions. The weighted means of TNF-α induction activity ranged from 4.3 to 6.6 EU/ml (Fig. 2). Neither dose-dependent interference nor vaccine batch-related interference with the activity of the Bp-LPS by the DTaP vaccines was observed.

DTaP vaccine batches A and C to which Bp-LPS was added at a final concentration of 100 EU/ml and the control Bp-LPS containing 100 EU/ml were kept at 4 °C for a week. Residual LAL activity and TNF-α induction activity of the samples were measured and the activities of the vaccine preparations were calculated in reference to those of the control Bp-LPS. The LAL activity of DTaP vaccine batch

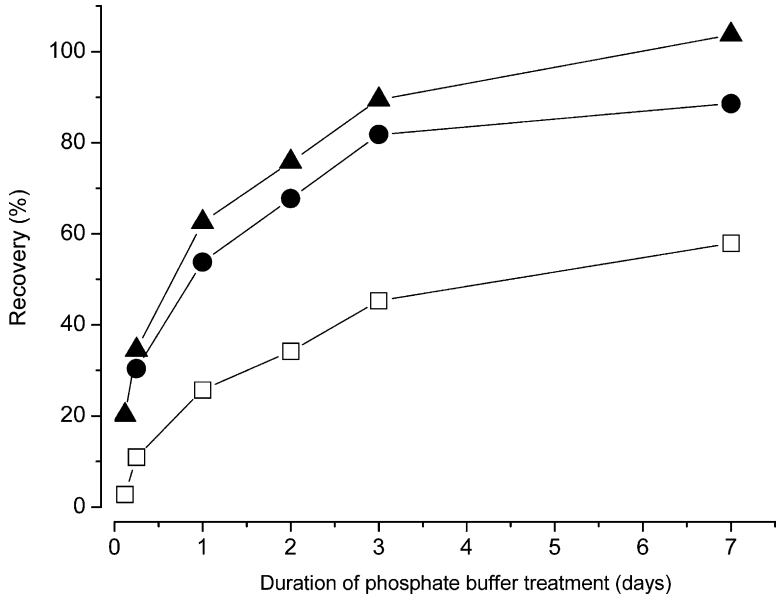

Fig. 1. Effect of phosphate buffer treatment to eliminate the interference of DTaP and aluminum hydroxide gel with LAL activity of LPS. LAL activity of Bp-LPS added to DTaP vaccine batches A (●) and B (▲), and 0.16% Al/ml of aluminum hydroxide gel (□) was measured just before addition of phosphate buffer (pH 8.0) at a final concentration of 0.25 M, and 6 h and 1, 2, 3 and 7 days later.

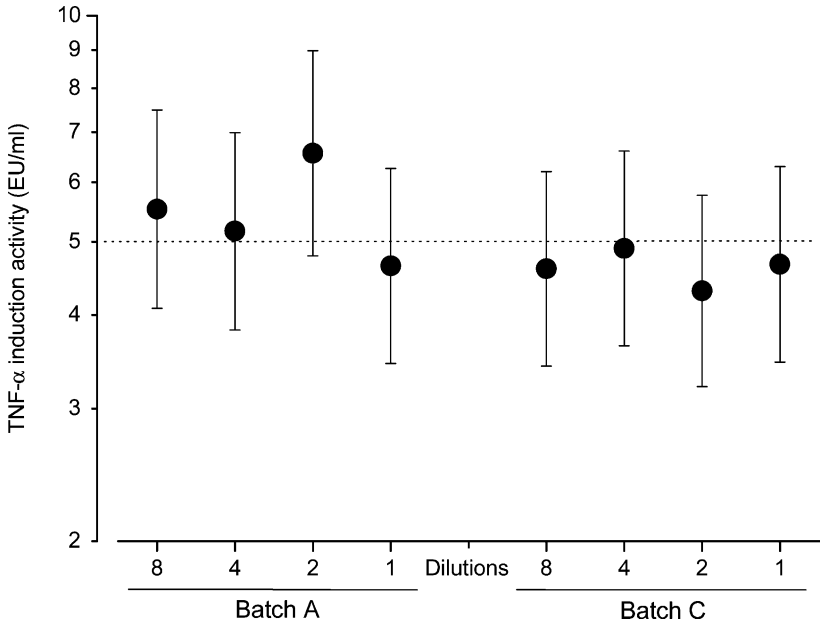

Fig. 2. Effect of DTaP vaccine dilutions on TNF-α induction activity of LPS. Two DTaP vaccines were serially diluted at two-fold intervals and added Bp-LPS to be 5.0 EU/ml. The measurement was carried out three times. TNF-α induction activity of the added Bp-LPS to DTaP vaccine preparations was calculated in reference to that of the control Bp-LPS. Each point shows the weighted mean of TNF-α induction activity of the added Bp-LPS. The vertical bars show 95% confidence intervals.

Table 2
Effect of DTaP vaccines on TNF-α induction of LPS in rabbit blood

Samples	Added LPS (EU/ml)	LAL test		TNF-α induction[a]	
		EU/ml	Recovery (%)	EU/ml	Recovery (%)
DTaP batch C	0	Negative	–	Negative	–
DTaP batch A	0	Negative	–	Negative	–
Distilled water + LPS[b]	100	83.9	100	88.1 ± 22.2	100
DTaP batch C + LPS[b]	100	104.6	124.7	82.6 ± 23.4	98.3 ± 12.6
DTaP batch A + LPS[b]	100	31.7	37.8	67.2 ± 16.8	77.1 ± 17.4

[a] TNF-α concentration in the culture supernatant of rabbit blood at 5 h after stimulated with the samples was determined by an ELISA method. The results represent mean ± S.D. values for four repeated measurements.
[b] Kept at 4 °C for 1 week before measurements.

A was decreased to 37.8% of the control LPS, while the in vitro TNF-α induction activity of the vaccine preparation retained more than 75% of the activity (Table 2).

4. Discussion

The LAL test has been implemented for testing an endotoxin contamination in various parenteral drugs including biological products [3,8–10]. The LAL test has been prescribed also in the WHO guidelines for acellular pertussis vaccines to test residual endotoxin [11].

We evaluated the applicability of the LAL test to DTaP vaccines as a substitution for the current BWD toxicity test. In the course of the evaluation, we found that some DTaP vaccine batches strongly interfered with the LAL test and the interference progressed gradually during a storage at 4 °C [12], while in vivo biological activities such as pyrogenicity in rabbits [12] and lethal activity in D-galactosamine-treated mice (Table 1) were not affected by the DTaP vaccine batches. These findings might suggest that residual endotoxin in the final products of DTaP vaccines would lose its LAL activity in spite of retaining its in vivo biological activities. Although the LAL test has been prescribed in the WHO guidelines, special care would be necessary for ensuring effectiveness of the test in detecting endotoxin of DTaP vaccines.

We attempted to find a way to eliminate the interfering effect of DTaP vaccines on the LAL activity of endotoxin. It was suggested that Al-hydro gel can be a major causal factor of the interference, because Al-hydro gel alone markedly interfered with LAL activity of endotoxin [12]. The treatment with a chelating agent, sodium citrate, was examined to eliminate the interference of the DTaP vaccine,

but was not effective. LAL activity in the vaccine preparations increased by the treatment with 0.002, 0.01 and 0.05 M phosphate buffer at 4 °C, but the recovery was only about 40–45 and 50–55% after 1 and 4 weeks, respectively (data not shown). The treatment with 0.25 M phosphate buffer was effective in eliminating almost completely the interfering effect but the recovery of the LAL activity was gradual (Fig. 1). The mechanism of the elimination of the interference by the phosphate buffer treatment could not be clarified in the present study. However, DTaP vaccines (e.g. batches A and B) of which buffer solution was acetate buffer interfered with the LAL activity, while DTaP vaccines (e.g. batch C) containing phosphate-buffered saline as buffer solution did not. These facts suggest that the presence of phosphate buffer would be an important factor to eliminate the interfering effect on the LAL activity of endotoxin in DTaP vaccines. But the eliminating effect of phosphate buffer was not affected by replacement of the buffer solution of the vaccine preparation with distilled water or 15 mM acetate buffer, pH 6.0 (data not shown).

We reported previously a novel test method for quantitative detection of endotoxin activity using peripheral blood of rabbits [13]. The test method was assumed to have a number of advantages [13]. The test could detect the biological activity, which was closely correlated with pyrogenicity in rabbits compared to the LAL test. The test is much more sensitive and accurate and requires reduced number of animals compared to the in vivo tests such as the pyrogen test, the BWD toxicity test and the lethal activity test using D-galactosamine-treated mice. We, therefore, examined the applicability of the in vitro TNF-α induction assay to DTaP vaccines. No dose-dependent cytotoxicity on the rabbit blood cells by DTaP vaccines was observed in the production of TNF-α in response to the stimulation with endotoxin (Fig. 2). Endotoxin of which LAL activity was interfered in DTaP vaccines during a storage at 4 °C, while retained the similar level of TNF-α induction activity to that of the corresponding control endotoxin (Table 2). Accordingly the in vitro TNF-α induction assay was considered as an effective assay method to detect quantitatively residual biological activity of endotoxin in DTaP vaccines.

However, we examined only Japanese DTaP vaccines in the present study, it is not clear yet whether the phosphate buffer treatment can be effective in eliminating the interfering effect of DTaP vaccines other than Japanese vaccines, if any, and the in vitro TNF-α induction assay can also be an effective assay to detect endotoxin activity of the DTaP vaccines.

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