

Short Communication

Endotoxin Content in *Haemophilus influenzae* Type b Vaccine

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SUMMARY: *Haemophilus influenzae* type b (Hib) is a major cause of bacterial meningitis among children. Hib conjugate vaccines have effectively prevented Hib infection, and routine immunization with Hib conjugate vaccine has diminished the incidence of the disease in the United States and European countries. Introduction of Hib conjugate vaccines is also required in Japan. However, endotoxin that can carry over from Gram-negative *H. influenzae* with a purified component may contribute to adverse events following Hib vaccination. In the present study, we examined the endotoxin content in Hib conjugate vaccines. The Hib conjugate vaccine batches, which were produced by a European vaccine manufacturer, were shown to have considerably high endotoxin activity and to vary from 13.9 to 173.7 endotoxin units/dose. These results suggest that it is necessary to monitor the endotoxin content of the vaccine batches to ensure the quality and safety of the vaccines.

Haemophilus influenzae type b (Hib) was the leading cause of bacterial meningitis and other serious invasive diseases among infants in the United States (U.S.) and European countries before Hib conjugate vaccines with a carrier protein became available (1,2). The conjugate vaccines against Hib infection became commercially available in the late 1980s (3), and have effectively prevented the incidence of invasive Hib disease (4,5). Routine immunization of children with the conjugate vaccines has been widely introduced in the industrialized countries, while the vaccines are not currently used in Japan because of the lower incidence of Hib meningitis in children compared to that in the U.S. and European countries (1,2,6). Introduction of the Hib conjugate vaccines is, however, required in Japan because Hib is also a major cause of bacterial meningitis in children.

Lipopolysaccharide, which is also referred to as endotoxin constituting the cell wall of Gram-negative bacteria, is known to have various biological activities and to cause harmful physical effects such as a febrile reaction in humans even in very small amounts (7,8). Endotoxin content in parenteral drugs, therefore, has to be strictly controlled to guarantee their safety. Relative to this concern, the Hib vaccines could possibly be contaminated with endotoxin, which can be carried over from Gram-negative *H. influenzae* with polyribosylribitol phosphate (PRP) in the process of purification. We therefore examined the endotoxin content in Hib vaccines.

Five batches of Hib conjugate vaccine, which consists of PRP conjugated to tetanus toxoid, from a European vaccine manufacturer, were used in the present study.

Limulus amoebocyte lysate (LAL) activity of the Hib vaccine was measured by using an endotoxin-specific chromogenic LAL reagent (Endospecy, Seikagaku Corp., Tokyo) whose reactivity with (1-3)- β -D-glucan is completely removed (9,10). A 50 μ l-volume of each of the appropriate dilutions of Japanese national reference standard endotoxin (RSE) and

the vaccines in pyrogen-free distilled water was mixed with equal volume of the LAL reagent. Rate of color development due to the release of *p*-nitroaniline was measured using an incubator-equipped micro-plate reader (Well Reader SK603, Seikagaku Corp.). Endotoxin content in the vaccines was calculated according to the parallel line assay method (11) using logarithmic values of dose and the rate of color-development in reference to those of RSE, and expressed as endotoxin units (EU).

In vitro test (12), which utilized induction of prostaglandin E₂ (PGE₂) in rabbit peripheral blood, was carried out to examine the biological activity of the endotoxin. A 0.1 ml-volume of a dilution of endotoxin or samples and a 0.15 ml-volume of heparinized peripheral blood collected from female rabbits of the Japanese white strain (Kitayama Labes Co., Ltd., Nagano, or Japan Laboratory Animals, Inc., Tokyo) were gently mixed in a pyrogen-free centrifuge tube containing 0.75 ml of pyrogen-free saline. The mixture was incubated at 37°C for 8 h, and PGE₂ concentration of the supernatants was measured by a commercial enzyme immunoassay kit (High Sensitivity Prostaglandin E₂ Enzyme Immunoassay Kit, Assay Designs, Inc., Ann Arbor, Mich., USA) in reference to the PGE₂ standard (Assay Designs, Inc.).

Three female rabbits of the Japanese white strain (Kitayama Labes Co., Ltd., or Japan Laboratory Animals, Inc.) weighing approximately 3 kg were intravenously injected with 1.0 ml/kg of endotoxin dilutions or a sample. Rectal temperature of the rabbits was monitored for 3 h using an electric thermometer (Scanner Unit X115 with High Accurate Data Logger K730, TECHNOL SEVEN, Kanagawa), and the maximum temperature rise during 3 h after injection was measured.

In vitro PGE₂ induction activity and pyrogenicity were calculated to express as EU-equivalent using logarithmic dose and the optical density or the rise in temperature in reference to those of endotoxin, which has a known EU value, according to the parallel line assay method.

Five batches of Hib conjugate vaccine were shown to have varied levels of LAL activity ranging from 13.9 to 173.7 EU/dose (Table 1). No consistency of endotoxin content was found among the Hib conjugate vaccine batches tested. Measuring endotoxin content of the vaccines was, therefore, assumed to

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Table 1. LAL activity of Hib conjugate vaccines

Hib vaccine Batch	LAL activity (EU/dose)		Recovery percent of spiked endotoxin ¹⁾
	Mean	95% confidence interval	
A	164.2	139.1 – 193.5	103.4
B	173.7	137.5 – 220.4	104.9
C	79.3	62.4 – 100.1	97.1
D	22.5	17.8 – 28.4	94.3
E	13.9	10.9 – 17.7	91.3

¹⁾: Japanese reference standard endotoxin was spiked to each Hib vaccine batch.

be necessary for monitoring batch-to-batch variation. We quantified the in vitro PGE₂ induction activity and the in vivo pyrogenicity of endotoxin in Hib vaccine batch A. The Hib vaccine showed high levels of endotoxin activity in both the in vitro and in vivo tests, which findings were similar to those of the LAL test (Fig. 1). This suggests that such a Hib vaccine highly contaminated with endotoxin could cause a febrile reaction in vaccinees.

Hib vaccines are generally injected simultaneously with diphtheria-tetanus-pertussis combined (DTP) vaccines, separately or as a combined preparation. When Hib conjugate vaccines were introduced in the U.S. and European countries, the endotoxin in Hib vaccine could be considered a negligible quantity compared to that in the routinely implemented whole cell pertussis based combination (DTwP) vaccines, which contained endotoxin levels above 10,000 EU/ml. It was, therefore, assumed that no difference in the rates of febrile reaction might be seen between those who received DTwP vaccine alone and those who received DTwP vaccine together with Hib vaccine. Acellular pertussis based combination (DTaP) vaccines contributed to reduce febrile reaction following immunization (13) owing to the remarkable improvement of pertussis vaccines, from which endotoxin was almost completely removed by the purification processes. The safety level of a vaccine should be evaluated relatively, based on that of already implemented relevant vaccines, to avoid adversely impacting on the achieved level of safety. Accordingly, implementation of Hib vaccines with high endotoxin content may affect the situation regarding safety

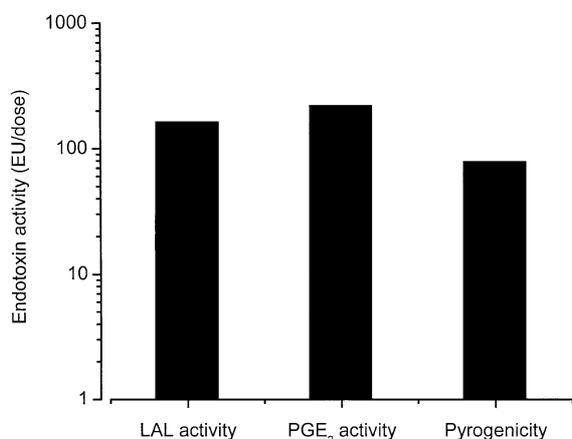


Fig. 1. Endotoxin activities of Hib conjugate vaccine. LAL activity, in vitro PGE₂ induction activity in rabbit peripheral blood and pyrogenicity were measured using vaccine batch A. The activities of the vaccine batch were evaluated in reference to those of endotoxin from *Bordetella pertussis* phase I Tohama strain, the LAL activity of which was calculated in reference to that of the Japanese national reference standard endotoxin.

in nations where DTaP vaccines have already been routinely used. The results suggest that the vaccine manufacturer could eliminate endotoxin from Hib conjugate vaccines to comparatively low levels of about 15 to 20 EU/dose. This effort is crucial also for an effective safety evaluation of clinical trials, which should be based on product constancy. It is, therefore, important to control residual endotoxin in the final products of Hib conjugate vaccines to ensure consistent safety of vaccinees.

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