Effects of Simultaneous Immunization of \textit{Haemophilus influenzae} Type b Conjugate Vaccine and Diphtheria-Tetanus-Acellular Pertussis Vaccine on Anti-Tetanus Potencies in Mice, Guinea Pigs, and Rats

Tadashi Fukuda\textsuperscript{1,2,*}, Masaaki Iwaki\textsuperscript{1}, Takako Komiyama\textsuperscript{1}, Keigo Shibayama\textsuperscript{1}, Motohide Takahashi\textsuperscript{1}, and Hideki Nakashima\textsuperscript{2}

\textsuperscript{1}Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo 208-0011; and \textsuperscript{2}Department of Microbiology, St. Marianna University of Medicine, Kawasaki 216-8511, Japan

(Summary: \textit{Haemophilus influenzae} type b vaccine conjugated with tetanus toxoid (HibT) was licensed for use in childhood immunization in Japan in 2007. As adsorbed diphtheria-tetanus-acellular pertussis (DTaP) combined with HibT vaccine has not been introduced in Japan, DTaP and HibT vaccines are injected at separate sites with a similar immunization schedule. There are various interfering or stimulatory effects between components of combined vaccines contained in DTaP and HibT vaccines. In this study, we investigated the effect of HibT containing combination vaccines on anti-tetanus potencies by using animal models (mouse, guinea pig, and rat). HibT vaccine and HibT components of imported DTaP-HibT vaccine alone showed comparable or higher anti-tetanus potency than DTaP vaccine and DTaP-containing components of combination vaccines. Mixing these components before injection resulted in potencies greater than the sum of individual potencies. Injecting individual components at separate sites in animals resulted in potency roughly equivalent to the sum of the individual potencies. These results provide useful information regarding the use of HibT-containing multivalent vaccines in childhood immunization.)

INTRODUCTION

\textit{Haemophilus influenzae} type b conjugate vaccine (Hib vaccine) consists of polyribosylribitol phosphate (PRP) from the outer membrane of \textit{H. influenzae} type b (Hib) and a carrier protein (e.g., tetanus toxoid, diphtheria toxoid-like protein [CRM197], or outer membrane protein of \textit{Neisseria meningitidis}) to enhance the immunogenicity of PRP (1–4). The vaccine is effective for the prevention of meningitis caused by Hib in childhood (5) and is used worldwide (1,5). In Japan, Hib vaccine containing tetanus toxoid as a carrier protein (HibT vaccine) was licensed for use in childhood immunization in 2007. Hib vaccine has an immunization program similar to that for diphtheria-tetanus-acellular pertussis combined vaccine (DTaP vaccine) (5,6). However, the immunization in childhood with Hib and DTaP vaccines has been complicated. Thus, DTaP-Hib vaccine is used in many countries to simplify immunization and reduce vaccination costs (6–8). However, DTaP-Hib vaccine has not yet been introduced for use in Japan. Thus, DTaP and HibT vaccines are used for primary immunization in childhood at ages 2, 4, and 6 months with an interval of 4 to 8 weeks, and the final dose in the series should be administered at age $\geq$ 12 months, on each simultaneously at different sites.

Previous studies indicate that vaccine antigens in many combined vaccines interfere with the immunogenicity of each other (8–10). Watemberg et al. reported a remarkable increase of anti-tetanus antibody titer in childhood given diphtheria-tetanus-whole-cell pertussis (DTwP)-HibT vaccine compared to those given DTwP vaccine alone (11). Using mouse and guinea pig models, Shams and Heron reported that HibT vaccine itself is comparably potent to DTaP-inactivated polio (IPV) vaccine (3). They also concluded that the combination of HibT and DTaP-IPV vaccines significantly increases anti-tetanus potency in mice and guinea pigs when the 2 vaccines are mixed before injection or injected at separate sites. HibT vaccine was introduced for use in Japan in 2007, and DTaP-IPV vaccine will be introduced soon. In this study, we evaluated the anti-tetanus potency of HibT vaccine using mouse, guinea pig, and rat models. The stimulatory effects of mixing HibT vaccine with DTaP vaccine or the DTaP-containing component of multivalent vaccines were investigated. The potencies of vaccine combinations were compared with those obtained by simultaneous injection at separate sites.

MATERIALS AND METHODS

Vaccine preparation: The Japanese vaccines used in this study were purchased from the commercial sources listed below: DTaP (2 lots; Kitasato Institute Research Center for Biologicals [currently, Kitasato Daiichi Sankyo Vaccine Co., Ltd.], Saitama, Japan), Hib (1 lot; Daiichi Sankyo Co. Ltd., Tokyo, Japan), and DTaP-IPV-HibT (3 lots; Sanofi Pasteur SA, Lyon, France).
HibT vaccine produced by Sanofi Pasteur SA and distributed by the Kitasato Institute Research Center for Biologicals is formulated to contain 10 μg of Hib PRP covalently bound to 24 μg of tetanus toxoid as a carrier protein in 0.5 mL of HibT vaccine (12). The DTaP-IPV-HibT vaccine by Sanofi Pasteur SA consists of DTaP-IPV liquid vaccine and lyophilized HibT vaccine components and is formulated to contain 5 Lf tetanus toxoid as a protective antigen against tetanus, 1.5 mg aluminum phosphate, and 10 μg Hib PRP covalently bound to 24 μg tetanus toxoid as a carrier protein per human dose (0.5 mL) (13). DTaP produced by the Kitasato Institute Research Center for Biologicals is formulated to contain <2.5 Lf tetanus toxoid and 0.90 mg aluminum chloride in 0.5 mL HibT vaccine (14).

The Japanese reference tetanus toxoid vaccine (Lot 2, 40 U/vial, 2.5 Lf/vial) (15) was used as a national standard preparation for the anti-tetanus toxoid potency test.

**Anti-tetanus toxoid potency test:** The anti-tetanus toxoid potency tests were performed as described by the Japanese Minimum Requirements for Biological Products (16). Serial 2-, 2.4-, and 3-fold dilutions in saline were used to immunize mice, guinea pigs, and rats, respectively; 3 or 4 doses were used for the reference, and 4 were used for the test vaccine preparations. Appropriate dilutions of each test vaccine and the reference were selected to obtain adequate dose responses with respect to tetanus toxoid antigen content. Mice (Slc:ddY, SPF, female, 22–24 g body weight, 5 weeks old, 10/group), guinea pigs (Slc:Hartley, SPF, female, 280–300 g body weight, 5 weeks old, 5/group), and rats (Slc:SD, SPF, female, 4 weeks old, 5/group) were inoculated subcutaneously in the lumbar region with 0.5 mL, 2 mL, and 0.5 mL of each dilution, respectively. Thirty-two days after immunization, the mice, guinea pigs, and rats were challenged by subcutaneous injection of 0.5 mL, 1 mL, and 0.5 mL tetanus toxin solution containing approximately 200 mice, 50 guinea pigs, and 200 rats LD50/mL, respectively. The time of death and symptoms of the challenged mice and guinea pigs were observed daily for 4 days and those of rats for 7 days.

The time of death and symptoms of the challenged mice and guinea pigs were converted to scores (Table 1) according to Ipsen (17) and Murata et al. (18) with some modifications (19). The scores were set such that the mean scores of the challenged animal groups would present an approximately linear regression when plotted against the log-transformed vaccine dose. The potencies of the test vaccines were determined by statistically analyzing scores using the parallel line assay method (20,21).

In the case of rats, the observation period for death was 7 days. The potencies of the test vaccines were determined by statistically analyzing the time of death using the probit assay method.

### RESULTS

**Anti-tetanus potency of HibT vaccines:** The anti-tetanus potencies of HibT vaccine and the Japanese DTaP vaccine were compared in various experimental animal models. The potential effects of DTaP vaccine on the anti-tetanus potency of HibT vaccine administered separately or as a mixture were also investigated.

A total of 3 lots of HibT component were used in this study. As shown in Table 2, the 3 lots of HibT component, each supplied as components of DTaP-IPV-HibT vaccine, exhibited 1539, 777, and 1098 units of anti-tetanus potency per mL. In contrast, the corresponding DTaP-IPV vaccines (Table 2; Lots A, B, and C) for each lot of HibT component exhibited 284, 373, and 81 units of potency, respectively. For all lots investigated, the HibT components showed greater anti-tetanus potency than their corresponding DTaP-IPV components. Upon mixing with the counterparts (i.e., dissolving the HibT components with DTaP-IPV components), Lots A, B, and C showed estimated anti-tetanus potencies of 5895, 6869, and 4649 units, respectively. The Japanese DTaP vaccine showed 111 units alone and reached 1855 units when mixed with the HibT component of the multivalent vaccines (Lot C). The anti-tetanus potency of the HibT component was also demonstrated in guinea pigs (Table 2); the HibT component of the Lot C vaccine exhibited anti-tetanus potency comparable to its corresponding DTaP vaccine.

**Anti-tetanus potency of DTaP plus HibT vaccines injected separately or after mixing:** We investigated whether mixing influences the overall anti-tetanus potency of HibT-containing combined vaccines by comparing them with separate injections of DTaP and HibT vaccines using products commercially available in Japan. Table 3 shows the overall anti-tetanus potencies of DTaP plus HibT vaccine injected separately and after mixing.

### Table 1. The scores assigned to the time of death and the symptoms of the challenged animal

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Score</th>
<th>Mouse</th>
<th>Guinea pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2nd day</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3rd day</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4th day</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Severe tetanus1</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mild tetanus2</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>No tetanus3</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

1: Severe tetanus: difficulty of moving, tonic spasm or respiratory distress.
2: Mild tetanus: local spasm of the abdominal muscle in opposite side to the site of injection, or animal’s body bends to the injected side.
3: No tetanus: no specific symptoms. 

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Table 2. The anti-tetanus potency of the commercial multivalent vaccines and each component using mouse and guinea pig

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Lot no.</th>
<th>Component</th>
<th>Potency [95% confidence interval]</th>
<th>Mouse</th>
<th>Guinea pig</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HibT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HibT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HibT</td>
<td></td>
<td></td>
<td>87 [40–244]</td>
</tr>
<tr>
<td>DTaP</td>
<td>D</td>
<td></td>
<td>111 [58–198]</td>
<td></td>
<td>107 [68–179]</td>
</tr>
<tr>
<td>DTaP-HibT</td>
<td></td>
<td>D + C</td>
<td>1855 [1169–3091]</td>
<td>634 [273–2285]</td>
<td></td>
</tr>
</tbody>
</table>

1: DTaP-IPV-HibT vaccine reconstituted freeze-dried HibT vaccine component with liquid DTaP-IPV vaccine component.
2: DTaP-IPV vaccine component of DTaP-IPV-HibT vaccine.
3: HibT vaccine component resolved with 0.017 M phosphate buffered sodium chloride solution containing 0.2 w/v gelatin (pH 7.0).
4: HibT vaccine component of DTaP-IPV-HibT vaccine Lot C, reconstituted with DTaP vaccine Lot D.

Table 3. Influence of HibT vaccine on anti-tetanus potency of DTaP vaccine in various experimental animals

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Lot no.</th>
<th>Immunization</th>
<th>Potency (U/mL)</th>
<th>Mouse</th>
<th>Guinea pig</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>HibT</td>
<td>F</td>
<td>single</td>
<td>779.4 [383.6–3512.3]</td>
<td>55.5 [17.8–96.3]</td>
<td>156.4 [33.0–631.1]</td>
<td></td>
</tr>
<tr>
<td>DTaP + HibT</td>
<td>E + F</td>
<td>mix</td>
<td>1378.2 [735.4–3498.3]</td>
<td>235.4 [130.6–375.2]</td>
<td>1628.0 [574.7–5281.7]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>separate</td>
<td>942.3 [488.3–2034.7]</td>
<td>156.3 [73.2–250.6]</td>
<td>744.1 [74.1–11946.2]</td>
<td></td>
</tr>
</tbody>
</table>

1: 95% confidence interval.
2: HibT vaccine resolved with DTaP vaccine was inoculated.
3: DTaP vaccine and HibT vaccine were simultaneously immunized to another site.

and rats, suggesting that the enhancement of anti-tetanus potency is not species-specific and that such effects can be avoided by separate injection.

**DISCUSSION**

The results of the experimental animal models indicate that the tetanus toxoid component used as a protein carrier in HibT vaccine is itself a potent immunogen for protection against tetanus, which is consistent with the results of Shams and Heron (3). In addition, mixing HibT vaccine with DTaP or DTaP-IPV vaccines increases the anti-tetanus potency to greater than the sum of the potencies of the individual components (i.e., HibT and DTaP vaccines). In some cases, the degrees of stimulation observed in our study were greater than those reported by Shams and Heron. In the present study, the separate injection of HibT and DTaP, and HibT and DTaP-IPV vaccines did not result in such synergistic effects on anti-tetanus potency.

In a number of countries including members of the European Union, the USA, and Asian countries, DTaP and Hib vaccines are administered to children in a combined form as DTaP-Hib vaccine. In contrast, DTaP-Hib vaccine has not yet been introduced in Japan. Instead, in Japan, DTaP and Hib vaccines are given simultaneously at different injection sites in childhood. A recent post-marketing survey in Japan shows that HibT vaccine does not affect the tetanus antitoxin titer of DTaP vaccine in childhood immunization when injected separately into opposite arms (22). Similar results are reported in India using DTaP and HibT vaccines (23). Thus, the results of the present animal models corroborate the benefit of separate injections for human Hib immunization.

Tetanus toxoid is included in HibT vaccine as a carri-
er protein for Hib PRP (24). The tetanus toxoid content usually reaches ≥20 μg/human dose, which is comparable to the amount contained in DTaP vaccine for tetanus immunization (3,6,24). Therefore, it can be assumed that this amount of toxoid is comparably immunogenic to DTaP vaccines in humans. We demonstrated that the anti-tetanus potency of HibT vaccine and enhancement of the anti-tetanus potency in HibT-DTaP vaccine mixture in experimental animals are comparable. Animal potency tests are widely used in vaccine quality control and are mandatory according to the WHO Minimum Requirement (25), highlighting that the WHO recognizes that animal potency tests are at least in part a suitable indication for estimating human immunogenicity. Therefore, the results of the present study corroborate the importance of animal potency testing required in vaccine quality control.

However, further studies are required to confirm the present experimental results—namely, whether the differences between the potencies of vaccines injected at separate sites and after mixing reflect any presently unknown effects in humans.

DTaP vaccine contains aluminum adjuvant to enhance tetanus and diphtheria toxoid potency (26,27). By themselves, HibT vaccines, which possess tetanus toxoid antigen levels above or comparable to those of DTaP vaccines, are aluminum adjuvant-free products. However, the immunogenicity of the tetanus toxoid antigen in HibT vaccine could be enhanced upon mixing with DTaP or DTaP-IPV vaccines, which contain aluminum adjuvant. The degree of these reactions varies between animal species. In the present study, aluminum-containing HibT vaccines exhibited enhanced anti-tetanus potency in mice, which was more pronounced than that in guinea pigs and rats. In 1957, mice were shown to be more sensitive to anti-tetanus potency enhancement by aluminum adjuvant than guinea pigs (28). This may corroborate the present observation that mice exhibited greater enhancement than guinea pigs and rats. Such species-dependent differences in interference are also reported with multi-component vaccines (8). Elucidating the mechanisms underlying the stimulation of potency in animal experiments will contribute to the development of a suitable quality control system for HibT and DTaP-Hib vaccines, which will be introduced for human use in Japan in the near future.

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Conflict of interest  None to declare.

REFERENCES


