Biotechnology and Bioprocess Engineering **2004**, 9: 490-494 

# An Animal Model to Evaluate the Protective Efficacy of *Haemophilus influenzae* Type b Conjugate Vaccines

**Hyun Sung Kim[1,2]*, Tae Hyeon Yoo[1], Yang Suk Jang[1,3], Hun Kim[1], Jin Yong Park[1,3], Byung-Ki Hur[2], Yeon-Woo Ryu[3], and Jong Su Kim[1]**

[1] GreenCross Vaccine Corporation, Yongin 449-903, Korea
[2] Department of Biotechnology and Bioengineering, Inha University, Incheon 402-751, Korea
[3] Department of Molecular Science and Technology, Ajou University, Suwon 442-749, Korea

**Abstract** An efficacy test of PRP (polyribosylribitol phosphate)–TT (Tetanus toxoid) conjugate vaccines was carried out using BALB/c mice as an animal model by inoculating *Haemophilus influenzae* type b (Hib) with a virulence enhancement factor (VEF). Three administrations of the conjugate vaccines at 2-week intervals elicited a significantly high level of PRP antibodies ($P < 0.0001$). The protective activity of the PRP immunization was challenged with either Hib with iron dextran (Hib*i*) or with a combination of mucin and hemoglobin (Hib*mh*) as a VEF. The medium lethal dose ($LD_{50}$) for Hib*mh* and Hib*i* was measured as 10 CFU (Colony Forming Unit) and $2.5 \times 10^8$ CFU respectively. Each immunized animal was challenged with five or ten times the $LD_{50}$ level of bacteria with a VEF. A significant difference in mortality between the immunized and control mice ($P < 0.01$) was observed with the Hib*mh* challenge inoculation but not with the Hib*i* challenge inoculation. These results show that a combination of mucin and hemoglobin was able to enhance the virulence of Hib in BALB/c mice to cause a lethal infection, thus suggesting that BALB/c mice introduced to this method can be an effective model animal for testing the protective efficacy of *H. influenzae* conjugate vaccines.

*Keywords*: *Haemophilus influenzae* type b, conjugate vaccine, challenge, virulence enhancement agent, combination of mucin and hemoglobin, efficacy

## INTRODUCTION

*Haemophilus influenzae* type b (Hib) has been a leading cause of bacterial meningitis among infants and young children worldwide. This organism also causes other invasive infections, including epiglottitis, cellulites, pneumonia, pericarditis, arthritis, bacteremia, empyema, and osteomyelitis [1-3]. United States Public Health officials estimate that 1 in 2,000 children contract Hib disease before the age of 5 years in the United States [4]. Pittman [5] demonstrated that the Hib capsular polysaccharide was an important toxic substance and antibody to the type b capsule conferred type-specific protection against lethal experimental Hib infection in animals. The bacterial polysaccharide is the important substance that can be developed industrially [6]. A purified polysaccharide (PRP) vaccine was developed industrially in the 1970s, but it was recommended only for children over 18 months of age because of its T-cell independence [7]. PRP-protein conjugate vaccines overcome this limitation of the polysaccharide vaccine and have been shown to provide protection from invasive Hib disease in infants [8].

The development of a successful vaccine can be achieved by evaluating the efficacy of candidate vaccines through an efficient and reliable animal model that tests an infection. The lack of an appropriate animal model for a bacterial infection has hampered the research of developing effective immunizing agents. Several animal models have been tested for the study of Hib disease, such as mice infected intraperitoneally with bacteria with or without enhancement [9,10], and infant rats infected intraperitoneally [11] or intranasally [12]. However, each of these models has the limitation that a large number of bacteria are often required. Thus, a new animal model using a combination of mucin and hemoglobin as a VEF was proposed [13] and modified [14]. The capacity of several VEFs was compared, and among them, iron dextran showed the best results [15].

Infant rats have been used for the evaluation of the protective efficacy of PRP polysaccharide vaccines by intranasal inoculation of the bacteria [16]. However, the protective efficacy evaluation of Hib conjugate vaccines using animal models has not been reported. In this study, we explored the effectiveness of a BALB/c mice model challenged by Hib with a virulence enhancement factor for testing the efficacy of Hib conjugate vaccines. Iron dextran and a combination of mucin and hemoglobin

***Corresponding author**
Tel: +82-31-280-6187 Fax: +82-31-280-6229
e-mail: hskim7@greencrossvaccine.com

were selected based on previously published results [15, 17].

## MATERIALS AND METHODS

### Animals

Pathogen-free inbred BALB/c mice were obtained from Charles River Japan Inc., Japan. All animals weighed 18~20 g at 4~5 weeks of age and were housed under standard conditions of temperature (21~25°C) and relative humidity (50 ± 10%) with a 12 h lighting schedule. The air recycling rate was between 10 and 15 times per hour. The lighting strength was 150~300 Lux. Food and water were available *ad libitum*. The inoculated and uninoculated animals were housed separately at 10 pups in each vivarium for the prevention of cross-infection by aerosol.

### Immunization

One PRP-TT conjugate vaccine (Hibvax) was prepared at the Vaccine R&D center of GreenCross Vaccine Corporation, Korea, and the other vaccine (Hiberix) was obtained from Glaxo Smith Klein, Belgium. Each dose of 0.25 mL containing 2.5 μg PRP-TT was administered three times into each group of 10 mice *via s.c.* at a 14 day interval. On day 35 the immunized mice were exsanguinated after ether anesthesia or were challenged by Hib with a VEF.

### Antibody Assay

PRP antibodies were measured by an enzyme-linked immunosorbent assay (ELISA) according to Kristensen and Bentzon [18] with some modification. Mouse hyperimmune PRP antiserum was calibrated with 70 μg/mL of the reference PRP antibody (Food Drug Administration, USA) by a radioimmunoassay [19,20] and used as a reference for ELISA. Microwell plates (Nunc, Denmark) were coated overnight at 4°C with tyraminated PRP in 50 mM $NaHCO_3$, pH 9.6 (0.1 μg PRP/mL). The plates were washed four times with PBS containing 0.1% Tween-20 (PBS-T). Eight two-fold dilutions of the sera in PBS-T were added and after a 2 h incubation at 37°C the plates were washed four times with PBS-T. Peroxidase-conjugated sheep anti-mouse immunoglobulin (Amersham Bioscience, Germany) diluted to 1:1,000 in PBS-T was added. After 1 h incubation, the washing was repeated four times. The plates were developed with a substrate (30 mg of orthophenylendiamine in 75 mL of 0.1 M phosphate buffer, pH 6.0), to which 100 μL of 35% $H_2O_2$ was later added immediately before use. After 20 min the reaction was stopped with 1.6 N sulfuric acid. Optical density was measured at 490 nm. Reaction volumes were 100 μL in all steps. The antibody titer of each serum was calculated from the standard curve of the reference antibody using the 4 parameter analysis and summarized as the geometric mean of μg of PRP antibody/mL.

### Virulence Enhancement Factors

Iron dextran (Sigma Chemical Co., USA) was diluted to 10 mg/mL in sterile saline. Mucin (Sigma Chemical Co., USA) and hemoglobin (Becton Dickinson and Company, USA) were dissolved in distilled water at 10% and 4% respectively, and the solution was sterilized by gamma irradiation at 1.5 Mega Rads.

### Bacteria

The Hib strain was obtained from the American Type Culture Collection, USA (No. 31441). The bacteria were grown overnight at 37°C in an atmosphere of 5% $CO_2$ on chocolate agar slants supplemented with 1% Isovitalex (BBL Microbiology Systems, Cockeysville, MD, USA).

### Infection

The bacterial colonies on the chocolate agar slants were scraped and suspended in brain heart infusion broth (BHI, Becton Dickinson and Company, USA) and the concentration was adjusted to $10^{10}$ bacteria/mL spectrophotometrically. Serial dilutions were made in BHI and the actual cell concentrations were verified by colony counts. Mice, in groups of 10, received an intraperioneal inoculation of a serial dilution of *H. influenzae* type b with iron dextran or mucin combined with hemoglobin. Iron dextran (0.5 mL) and a serial dilution of bacteria (0.5 mL) were simultaneously injected [15] and mucin-hemoglobin (0.5 mL) and a serial dilution of bacteria (0.5 mL) were mixed together prior to inoculation [14]. The death rate of mice was recorded for 72 h and a cumulative mortality was calculated per group.

### Determination of $LD_{50}$

The $LD_{50}$, the dose that causes 50% mortality was estimated by the *probit* method [21].

## RESULTS AND DISCUSSION

### Serum PRP Antibodies in Mice

PRP antibody titers were measured by ELISA [18] and the results are shown in Table 1. A statistical comparison of antibody levels was done by the student *t*-test analysis. The injection of PRP-TT conjugate vaccines elicited a statistically significant rise of PRP antibodies ($P < 0.0001$), but there was no difference between the two conjugate vaccines from different sources. Despite the demonstration of the vaccine's immunogenicity, this result alone does not show any immunological characteristics of the elicited antibodies and host immune system. In particular, the protection against a pathogen is one of the most important goals of vaccine immunization. The current study proposes that the animal model, which challenged immunized mice with Hib with a virulence enhancement factor, evaluated the protective capability of PRP antibodies.

**Table 1.** Serum PRP antibodies of mice immunized with PRP-TT conjugate vaccines

| Immunogen | No. of mice | Geometric mean of μg of antibody/mL (25th~75th centiles)*a | *P* value*b |
|---|---|---|---|
| Hibvax | 20 | 4.65 (3.58~6.03) | < 0.00001 |
| Hiberix | 19 | 4.07 (3.11~5.34) | < 0.00001 |
| Saline | 17 | 0.093 (0.083~0.104) | - |

*a: Comparison of the geometric means were performed with the two-sided *t* test analysis.

*b: Values determined by the chi-square test for each immunogen group versus the saline group. Both PRP-TT conjugate vaccines elicited higher levels of anti-PRP compared to saline.

**Table 2.** Susceptibility of BALB/c mice to various VEFs increased *H. influenzae* type b infection capacity

| Strain of Hib | Mouse strain | Virulence enhancing factor | | $LD_{50}$(CFU) |
|---|---|---|---|---|
| | | Type | Concentration | |
| ATCC 31441 | BALB/c | None | - | $>10^{10}$ |
| | | Iron dextran | 5 mg/mL | $2.5 \times 10^8$ |
| | | Mucin-Hemoglobin | 5% and 2% | 10 |
| Strain No 12*a | BALB/c | Iron dextran | 5% | $10^5$ |
| | | Mucin-Hemoglobin | 5% and 2% | $3.0 \times 10^2$ |
| | SW/NIH*b | Mucin-Hemoglobin | 5% and 2% | $7.5 \times 10^3$ |

*a: These data were referenced to Brodeur *et al.* (1985) [17].
This strain was obtained from St. Justine Hospital, Montreal, Canada.

*b: Swiss Webster/NIH (SW/NIH)

**Table 3.** Rate of mortality in BALB/c mice challenged with *H. influenzae* type b after the immunization with PRP-TT conjugate vaccines

| Virulence enhancement factor | Inoculating dose (CFU/mouse) | Survival rate (%) at 72 h post-infection *a | | |
|---|---|---|---|---|
| | | Group 1*b | Group 2*c | Group 3*d |
| Iron dextran | $1.25 \times 10^9$ | $40^{v1}$ | $40^{v2}$ | $10^{v3}$ |
| | $2.5 \times 10^9$ | $10^{x1}$ | $20^{x2}$ | $0^{x3}$ |
| Mucin-hemoglobin | 50 | $90^{y1}$ | $90^{y2}$ | $10^{y3}$ |
| | 100 | $60^{z1}$ | $50^{z2}$ | $0^{z3}$ |

*a: Comparison of the mortality rates was performed with the *chi*-square test analysis.
Values of $P < 0.05$ were considered significant,
v1 or v2 versus v3 $P > 0.05$,
x1 versus x3 $P > 0.05$,
x2 versus x3, $P > 0.05$,
y1 or y2 versus y3, $P < 0.0005$,
z1 versus z3, $P < 0.005$,
z2 versus z3, $P < 0.01$

*b: Each Group 1 consisted of 10 mice immunized with Hibvax (2.5 μg of PRP per mouse, GCVC) on day 0, 14, and 28, and the mice were inoculated intra-peritoneally with Hib with enhancement factors on day 35.

*c: Group 2s were treated with the same method as Group1 except for the use of immunogen and Hiberix (2.5 μg of PRP per mouse, GSK)

*d: Group 3s were treated with the same method as Group1 except for the use of immunogen and saline.

### Virulence Enhancing Factor

To investigate the effect of several virulence enhancement vehicles on the virulence of Hib in mice, we examined the $LD_{50}$ for the different enhancement media. As shown in Table 2, the incorporation of mucin-hemoglobin to the inoculum greatly enhanced the virulence in BALB/c mice. For the Hib ATCC 31441 strain, the $LD_{50}$ of iron dextran and mucin-hemoglobin were $2.5 \times 10^8$ and 10 CFU, respectively. The virulence enhancement of mucin-hemoglobin was greatly higher than that of iron dextran in spite of the different mouse and Hib strains. However, the results from the different strains showed an insignificant difference between these virulence enhancements. In this study, the infection sizes were determined to be 5 and 10 times that of $LD_{50}$, respectively (Table 3).

No adverse symptoms were observed when only iron

dextran or mucin-hemoglobin was administered.

### Bacterial Challenge in Mice

For each VEF, 5 times or 10 times the amount of $LD_{50}$ bacteria were inoculated intraperitoneally into the immunized mice and the accumulated mortality was observed for 72 h (Table 3). Data were statistically compared by an analysis of proportions, using the *chi*-square analysis [20] and values of $P < 0.05$ were considered significant. In the case of iron dextran, a significant difference between the immunized and control mice was not observed ($P > 0.05$). However, the animal model using a combination of mucin and hemoglobin as a VEF showed the protective efficacy of the elicited PRP immunization. When 50 CFU bacteria were challenged, 90% of the mice immunized with Hibvax or Hiberix survived, but only 10% of the control mice survived ($P < 0.0005$). In the case of 100 CFU bacteria, the survival rate of the immunized mice was 60% for Hibvax and 50% for Hiberix, whereas 100% of the control mice died ($P < 0.01$).

In general, animal models that require a high concentration of bacteria for lethality may not be suitable to test the protective efficacy of vaccines. These models do not simulate the natural infection that usually is caused by a small dose of bacteria, and when a number of organisms are used, the "infection" is incidental to primary intoxication or endotoxemia [14]. Therefore, if a high quantity of bacteria were challenged at once, the protective efficacy of vaccines may not be evaluated appropriately. When iron dextran was used, it seemed that the animal model failed to resemble the natural infection of Hib, so the protective efficacy of the elicited PRP antibody could not be evaluated.

In the case of a combination of mucin and hemoglobin, the $LD_{50}$ was greatly reduced and a significant difference in the cumulative death rate between the immunized and the control mice group was observed. It has been suggested that a combination of mucin and hemoglobin interferes with the phagocytic process of the macrophages in the peritoneal cavity or systemic clearance, or in both, thus allowing organisms to enter the circulation [22,23]. Brodeur *et al.* [14] assumed that the reduced detoxification of endotoxin by these two agents may play a role in the effects observed.

An intranasal inoculation into infant rats was attempted in order to evaluate a PRP polysaccharide vaccine [16], but in this model the efficacy was only evaluated by a blood culture and cerebral spinal fluid analysis because of the resistance of mice to the bacteria. In addition, a nasal inoculation is very difficult to control and thus obtained results are not likely reproducible, which may hinder a correct evaluation of developed vaccines.

This current protective efficacy test was conducted *in vivo* and the protection rate was analyzed by a directly initiated Hib infection in immunized animals. Other potency tests of the Hib conjugate vaccine are the *in vitro* tests that were analyzed using the immunized serum. This efficacy test is a very specific test that is only applied to animals not to human infants. Specifically, an antibody concentration of over 1 μg/mL, in a serum obtained 1 month after a vaccination, was associated with long-term protection from Hib disease in human infants [24]. The alternative efficacy test used the antibody engineering has been developing at the various research labs [25]. For ethical and practical reasons, it is no longer feasible to perform randomized, placebo-controlled trials to assess the efficacy of a new Hib conjugate vaccine. Accordingly, scientists at the US Center for Biological Evaluation and Research, FDA, proposed the immunological criteria for evaluating new Hib conjugate vaccines for licensure in human infants [26]. These criteria were focused on to demonstrate the immunogenicity of a candidate vaccine comparable to that of a licensed vaccine, and they were applied subsequently to the licensure of the PRP-TT conjugate vaccine [27]. Therefore, performing an efficacy trial on human infants is not necessary [28]. In the current circumstance, the new efficacy test performed on mice may help confirm a candidated Hib conjugate vaccine at clinical test.

## CONCLUSION

This study shows that the protective efficacy of PRP-TT conjugate vaccines can be evaluated by challenging immunized BALB/c mice with Hib with a combination of mucin and hemoglobin. The animal model proposed in this study does not require other kinds of analyses (*e.g.* a nasal and intranasal inoculation, a blood culture and cerebral spinal fluid analysis) and protective efficacy can be evaluated just by inoculating the bacteria with a virulence enhancement factor. The simplicity of this method would not only reduce the total research time and efforts, but it would also improve accuracy and reproducibility.

This test method may be expanded to evaluate other types of Hib conjugate vaccines, *e.g.* PRP-CRM197, PRP-DT and PRP-OMP. Additional data are needed to establish the degree of correlation between human antibody responses and the prevention of bacteremia in the current animal model.

**Acknowledgement** This work was partially supported by Biotoxtech Company.

## REFERENCES

[1] Cochi, S. L., C. V. Broome, and A. W. Hightower (1985) Immunization of US children with *Haemophilus influenzae* type b polysaccharide vaccine. *J. Am. Med. Ass.* 253: 521-529.

[2] Coulehan, J. L., R. H. Michaels, K. E. Williams, D. K. Lemley, C. Q. Jr. North, T. K. Welty, and K. D. Rogers (1976) Bacterial meningitis in Navajo indians. *Public Health Rep.* 91: 464-468.

[3] Tarr, P. I. and G. Peter (1978) Demographic factors in the epidemiology of *Haemophilus influenzae* meningitis in young children. *J. Pediat.* 92: 884-888.

[4] Ward. J. (1991) Prevention of invasive *Haemophilus influenzae* type b disease: Lessons from vaccine efficacy trials. *Vaccine* 9: S17-24.
[5] Pittman M. (1933) The action of type-specific *H. influenzae* antiserum. *J. Exp. Med.* 58: 683-706.
[6] Onodera, M. (2002) Silicone rubber membrane bioreactors for bacterial cellulose production. *Biotechnol. Bioprocess Eng.* 10: 289-294.
[7] Immunization practices advisory (ACIP) (1986) Update: Prevention of *Haemophilus influenzae* type b disease. *Morbid. Mortal. Week. Rep.* 35: 170.
[8] Heath, P. T. and M. Fracp (1998) *Haemophilus influenzae* type b conjugate vaccines: A review of efficacy data. *Pediatr. Infect. Dis. J.* 17: S117-122.
[9] Burans, J. P., F. H. Kruszeski, M. Lynn, and M. Solotrovsky (1981) Kinetics of *Haemophilus influenzae* type b infection in normal and ribosom-immunized mice using intraperitoneal and intracerebral routes of inoculation. *Br. J. Exp. Pathol.* 62: 496-503.
[10] Kaplan, S. L., E. O. Mason, Jr., and B. L. Wiedermann (1983) Role of adherence in the pathogenesis of *Haemophilus influenzae* type b infection in infant rats. *Infec. Immun.* 42: 612-617.
[11] Smith, A. L., D. H. Smith, D. R. Averill, Jr., J. Marino, and E. R. Moxon (1983) *Haemophilus influenzae* type b bacteremia and meningitis infant rabbits after intranasal inoculation. *J. Lab. Clin. Med.* 102: 939-946.
[12] Moxon, E. R., A. L. Smith, D. R. Averill, and D. H. Smith (1974) *Haemophilus influenzae* meningitis in infant rats after intranasal inoculation. *J. Infect. Dis.* 129: 154-162.
[13] Marks, M. I., E. J. Ziegler, H. Douglas, and L. B. Corbeil (1982) Lethal *Haemophilus influenzae* type b infection in mice. *Infection* 10: 261-266.
[14] Broueur, B. R., Y. Larose, P. Tsang, J. Hamel, F. Ashton, and A. Ryan (1985) Protection against infection with *Neisseria meningitides* group B serotype 2b by passive immunization with serotype specific monoclonal antibody. *Infect. Immun.* 50: 510-516.
[15] Rodriguez, S. S., J. F. I. Bourzac, O. M. Chang, M. F. Medina, E. M. Carnago, and Y. L. Hernandez (1999) Virulence enhancement agents for *Haemophilus influenzae* type b infection in mice. *Lab. Anim. Sci.* 49: 95-98.
[16] Halesy, N. A., T. L. Johansen, L. C. Bowman, and M. P. Glode (1983) Evaluation of the protective efficacy of *Haemophilus influenzae* type b vaccines in an animal model. *Infec. Immun.* 39: 1196-1200.
[17] Brodeur, B. R., P. S. Tsang, J. Hamel, Y. Larose, and S. Montplaisir (1986) Mouse models of infection for *Neisseria meningitides* B, 2b and *Haemophilus influenzae* type b diseases. *Can. J. Microbiol.* 32: 33-37.
[18] Kristensen, K, and M. W. Bentzon (1992) Relation between enzyme-linked immunoserbent assay and radioimmunoassay for detection of antibodies to the capsular polysaccharide of *Haemophilus influenzae* type b. *APMIS* 100: 142-146.
[19] Farr, R. S. (1958) A quantitative immunochemical measurement of the primary interaction between I*BSA and antibody. *J. Infect. Dis.* 103: 239-262.
[20] Kuo, J. S. C., N. Monji, R. S. Schwalbe, and D. W. McCoy (1981) A radioactive antigen-binding assay for the measurement of antibody to *Haemophilus influenzae* type b capsular polysaccharide. *J. Immunol. Methods* 43: 35-47.
[21] Warlaw, A. C. (1985) *Practical Statistics for Experimental Biologists*. JohnWiley Wiley & Sons Ltd, USA.
[22] Corbeil, G. A., C. P. Kenny, and G. Laverne (1976) Iron as a replacement for mucin in the establishment of meningococcal infection in mice. *Can. J. Microbiol.* 22: 832-838.
[23] Miller, C. P. (1933) Experimental meningococcal infection of mice. *Science* 78: 340-341.
[24] Ward, J., G. Brenneman, G. W. Leston, and W. L. Heyward (1990) Limited efficacy of a *Haemophilus influenzae* type b conjugate vaccine in Alaska native infants. *N. Engl. J. Med.* 323: 1391-1401.
[25] Hong, H. J. and S. T. Kim (2002) Antibody engineering. *Biotechnol. Bioprocess Eng.* 7: 150-154.
[26] Frasch, C. E. (1994) Regulatory perspectives in vaccine licensure. pp.435-453. In: Ellis R. W, D. W. Granoff (eds.) *Development and Clinical Uses of* Haemophilus *b Conjugate Vaccine*. Mercel Dekkar, New York, USA.
[27] FDA approval of use of a new *Haemophilus* b conjugate vaccine and a combined diphtheria-tetanus-pertussis and *Haemophilus* b conjugate vaccine for infants and children. (1993) *Morb. Mortal. Wkly. Rep.* 42: 296-298.
[28] Granoff, D. M. (2001) Assessing efficacy of *Haemophilus influenzae* type b combination vaccines. *Clin. Infect. Dis.* 33: S278-287.

[Received August 31, 2004; accepted December 2, 2004]