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Development of a Quadrivalent Combined DTaP-HepB Vaccine with a Low Toxicity and a Stable HBsAg Immunogenicity

BAE, CHEON-SOON[1], KYUNG-NAM PARK[1], SANG-JEOM AHN[1], JONG-SU KIM[1]*, AND BYUNG-KI HUR[2]

[1]*Vaccine Institute, GreenCross Vaccine Corp., 227-3 Kugal-Ri, Kiheung-Eup, Youngin, Korea*
[2]*Department of Biotechnology and Bioengineering, Inha University, Incheon 402-751, Korea*

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Abstract When developing a combined DTaP-HepB vaccine, toxicity and HBsAg immunogenicity are both important considerations. Thus, for a combined DTaP-HepB vaccine with a low toxicity, the effect of the DTaP content and $Al(OH)_3$ gel concentration on the vaccine toxicity was investigated. Within the range studied, the higher the concentrations, the higher the vaccine toxicity. The importance of the tetanus toxoid content in the combined DTaP-HepB vaccine was also revealed. A higher concentration of the tetanus toxoid was found to have a negative effect on the stability of the HBsAg immunogenicity in the combined vaccine. Accordingly, considering the factors affecting toxicity and HBsAg immunogenicity, a novel DTaP-HepB vaccine (30 Lf/ml of diphtheria toxoid, 5 Lf/ml of tetanus toxoid, 10 μg PN/ml of acellular pertussis, 24 μg/ml of HBsAg, and 500 μg Al/ml of $Al(OH)_3$ gel) was developed. It has a low toxicity and a stable HBsAg immunogenicity and also satisfies the potency criteria of K-FDA for a combined DTaP vaccine.

Key words: *Immunogenicity*, combined vaccine, toxicity

A combined vaccine means a formulation that includes more than two immunogens within one preparation, for example, DTP and MMR against different diseases, and IPV and Rota against single diseases caused by multiple strains or serotypes of an infectious organism. Combined vaccines have many advantages, such as decreasing the number of immunizations, reducing the delivery cost, increasing the vaccine coverage, and the immunization of populations inaccessible due to geographic factors and/or individual tendencies. Therefore, many combined vaccines have already been developed and are expected to occupy more than 50% of the world vaccine market in the near future.

*Corresponding author
Phone: 82-31-280-6184; Fax: 82-31-280-6239;
E-mail: jskim3@greencrossvaccine.com

The current study developed a new formulation for a combined DTaP-HepB vaccine including diphtheria toxoid, tetanus toxoid, acellular pertussis antigens, and hepatitis B surface antigen (HBsAg). Mixing different monovalent vaccines can cause an immunogenicity reduction for more than one component due to the incompatibility of antigens as well as a toxicity enhancement by the increase in adjuvant content [1, 4, 14, 15, 17, 20]. In particular, a combined DTaP-HepB vaccine is known to have a low HBsAg immunogenicity by antigen incompatibility. The HBsAg incompatibility in a combined DTaP-HepB vaccine can be caused by impurities (enzymes, reducing agents, and so on) in the DTaP or failure in optimizing formulation. However, it is impossible to completely remove all impurities, because the diphtheria and tetanus toxoids used in the vaccine are produced by directly formalin-inactivating the culture media, including the toxin.

Accordingly, to overcome such limitations in a combined DTaP-HepB vaccine, we attempted to optimize a formulation. Diverse formulations with different contents of formulation factors such as DTaP content, tetanus toxoid content, and adjuvant content were prepared and compared for BWD toxicity, geometric mean anti-HBsAg titer, and potencies of DTaP components; thereby, clarifying factors affecting a BWD toxicity and HBsAg immunogenicity.

Finally, a new formulation of a combined DTaP-HepB vaccine was obtained, which not only showed a low BWD toxicity and a stable HBsAg immunogenicity for a long storage period of vaccine, but also satisfied the potency criteria of K-FDA for a combined DTaP vaccine.

Materials and Methods

Preparation of Toxoid Bulks, HBsAg Bulk, and Combined DTaP-HepB Vaccine

The diphtheria toxin excreted into a culture medium of the *Corynebacterium diphtheriae* Park Willium strain was

detoxified by formalin. This diphtheria toxoid was then purified by the combination of ultrafiltration, diafiltration, $(NH_4)_2SO_4$ precipitation, and gel permeation chromatography. The tetanus toxin excreted from the *Clostridium tetani* Harvard strain was formalin-detoxified, and purified by the method similar to the purification of the diphtheria toxoid. The pertussis proteins [PT(pertussis toxin), FHA (filamentous hemagglutinin), pertactin, and so on] were produced in a culture medium of *Bordetella pertussis*, co-purified by processes such as $(NH_4)_2SO_4$ precipitation, diafiltration, and microfiltration, and finally formalin-detoxified. The HBsAg secreted into the periplasmic space of recombinant *Hansenula polymorpha* was purified by sequential processes of cell disruption, ionic chromatography, ultrafiltration, diafiltration, ultracentrifugation, and gel permeation chromatography.

The diphtheria toxoid, tetanus toxoid, acellular pertussis, and HBsAg were separately adsorbed on an $Al(OH)_3$ gel, and the combined DTaP-HepB vaccine was prepared by mixing the adsorbed bulks in order of the diphtheria toxoid-absorbed, tetanus toxoid-adsorbed, acellular pertussis antigens-adsorbed, and finally the HBsAg-adsorbed bulk. The final pH of the combined DTaP-HepB vaccine formulations was adjusted to 6.5 by the addition of NaOH or HCl.

Immunogenicity Assay

The HBsAg immunogenicity and protective anti-HBsAg antibody rate (percentage of mice above 10 mIU/ml of protective anti-HBsAg IgG level) of the combined DTaP-HepB vaccine were measured using 30 SPF-grade ICR mice weighing 20–22 g. Each mouse was subcutaneously immunized with 0.5 ml of the combined DTaP-HepB vaccine and bled after 4 weeks. The anti-HBsAg IgG titer in each serum was assayed using the EIA method (Ausab IMx system, Abbott Laboratories, U.S.A.). From the assay results, the geometric mean (GM) anti-HBsAg IgG titer and protective anti-HBsAg antibody rate were then calculated.

For the immunogenicity of diphtheria toxoid, tetanus toxoid, and acellular pertussis, the potency was measured based on the following animal experiments (*the minimum requirements for biological products of Korea, K-FDA*). For the diphtheria potency (or tetanus potency), five guinea pigs weighing 300–400 g were separately immunized with 0.75 ml of the combined DTaP-HepB vaccine, bled by heart puncture after 35 days, and sera were pooled. Two fold- and four-fold-diluted antisera and a reference diphtheria antitoxin [1 IU/ml, NIBSC; reference tetanus antitoxin for tetanus potency, 1 IU/ml, NIBSC] were mixed with an equal volume of a reference diphtheria toxin (1L_f/ml, GCVC; reference tetanus toxin for tetanus potency, 1L_f/ml, GCVC). Next, two guinea pigs weighing 270–330 g (6 mice weighing 18±2 g were used for the tetanus potency) were each injected subcutaneously with 2 ml (0.2 ml for the tetanus potency) of one neutralized solution, then the neutralized antibody titer (diphtheria or tetanus potency) was calculated based on the death time of the animals.

The acellular pertussis potency of the combined DTaP-HepB vaccine was measured using SPF-grade ICR mice. The combined DTaP-HepB vaccine and reference vaccine (11.9 IU/ml, GreenCross Vaccine Corp., Korea) were diluted 8-, 40-, and 200-fold, respectively. Each dilution group was composed of 20 mice. All the mice were challenged into the brain with 0.025 ml of live *B. pertussis* 18323 (about 200 LD_{50}), 3 weeks after being intraperitoneally immunized with 0.5 ml of the diluted vaccine. Fourteen days after the challenge, the acellular pertussis potency was statistically calculated based on the number of surviving mice.

Mouse BWD Toxicity Assay

To obtain a calibration curve for measuring the mouse BWD (body weight decrease) toxicity of the combined DTaP-HepB vaccine, 0.5 ml of a BWD reference (102 BWD U/ml, GCVC, Korea) diluted 4-, 16-, and 64-fold was intraperitoneally injected into each dilution group, composed of 10 SPF-grade ICR mice. At the same time, 0.5 ml of the combined DTaP-HepB vaccine was injected through the same route into another 10 mice. Sixteen hours after the injection, the mice were weighed, and the mouse BWD toxicity (BWDU/ml) of the combined DTaP-HepB group was calculated from the calibration curve.

RESULTS

Effect of $Al(OH)_3$ Gel Concentration on Mouse BWD Toxicity

As important additives for enhancing the antigen immunogenicity, many adjuvants, such as alum gels, oil emulsions, complete Freund's adjuvant, lipopolysaccharide, saponin, liposomes, and biodegradable polymer microspheres, have already been developed, and alum gels like $Al(OH)_3$ and $AlPO_4$, have been largely used for human vaccines due to their low toxicity and extensive safety record [3, 5, 7, 12, 13].

Accordingly, in the current study, $Al(OH)_3$ gel was used as an adjuvant for the combined DTaP-HepB vaccine. However, in spite of its high safety, $Al(OH)_3$ gel can induce side effects when used in excess. Here, the toxicity of the combined DTaP-HepB vaccine was measured by a BWD (mouse body weight decrease) toxicity assay, as described in Materials and Methods. To study the correlation between the $Al(OH)_3$ gel concentration and the mouse BWD toxicity, three formulations of the combined DTaP-HepB vaccine with different alum concentrations were prepared (Alum content group in Table 1) and assayed for their BWD toxicity. The composition of antigens in the

Table 1. Composition of antigens and adjuvant in combined DTaP-HepB vaccines.

Group	Formulation	D (Lf/ml)	T (Lf/ml)	aP (μg PN/ml)	HBsAg (μg/ml)	$Al(OH)_3$ (μg Al/ml)
Alum content	I	50	15	12	24	350
	II	50	15	12	24	500
	III	50	15	12	24	800
DTaP content	IV	35	10.5	8.4	24	800
	V	42.5	12.8	10.2	24	800
	VI	50	15	12	24	800

D: diphtheria toxoid; T: tetanus toxoid; aP: acellular pertussis.

three formulations was fixed at 50 Lf/ml of the diphtheria toxoid, 15 Lf/ml of the tetanus toxoid, 12 μg PN/ml of acellular pertussis, and 24 μg/ml of HBsAg, and the $Al(OH)_3$ gel concentration was 350, 500, and 800 μg Al/ml, respectively (Table 1). The result show that the BWD toxicity proportionally increased from 13.6 to 34.2 BWD U/ml in the range of 350–800 μg Al/ml (Table 2). Therefore, this implies that the $Al(OH)_3$ gel concentration was one of the toxicity factors in the combined DTaP-HepB vaccine and should be kept as low as possible for a low toxicity.

Effect of DTaP Content on Mouse BWD Toxicity

The combined DTaP-HepB vaccine includes detoxified materials, such as diphtheria, tetanus, and pertussis toxoids, which were inactivated by the formalin-detoxification method, as described in Materials and Methods. However, there have been some reports that the formalin-detoxification method does not provide complete toxoiding [8, 11, 16]. Therefore, based on the concern that the DTaP content may have an effect on the toxicity of the combined DTaP-HepB vaccine, the following formulations were evaluated.

Three combined vaccines (DTaP content group in Table 1) with the same concentration ratio of DTaP antigens were formulated and assayed for their toxicities. These vaccines had different DTaP contents, which were 156, 193, and 236 μg/ml, respectively, while the alum concentration was fixed at 800 μg Al/ml. The animal study on these vaccines showed that the BWD toxicity increased from 16.6 to 34.2 BWD U/ml with a DTaP content of 156 to 236 μg/ml, thereby exhibiting a significant toxicity dependency on the DTaP content in the combined DTaP-HepB vaccine, as with the $Al(OH)_3$ gel (Table 2). Therefore, these results suggest that the DTaP content should be as low as possible to provide a combined DTaP-HepB vaccine with a low toxicity.

Effect of DTaP Content and Alum Concentration on the Immunogenicity of Combined DTaP-HepB Vaccines

The immunogenicities for the six formulations in Table 1 were assayed as described in Materials and Methods. It was found that the potencies of diphtheria, tetanus, and acellular pertussis were similar despite an increase in the alum gel and DTaP contents tried, whereas the anti-HBsAg IgG titer in serum was strongly dependent on the contents.

For the alum content, the GM anti-HBsAg IgG titer was 200, 390, and 470 mIU/ml with 350, 500, and 800 μg Al/ml of an $Al(OH)_3$ gel, respectively (Table 2). The adsorption rates for all the formulations in the current study were nearly 100%. In the case of the alum gel, which is known to induce humoral immunity by eliciting Th2-type responses, several studies have confirmed the necessity of free excess alum gel for higher adjuvant effect [2, 6, 9], in agreement with our present results, which revealed a proportional correlation between the alum gel concentration and the HBsAg immunogenicity.

Furthermore, it was also revealed that the DTaP content in the combined DTaP-HepB vaccine affected the HBsAg immunogenicity. The GM anti-HBsAg IgG titer was 280,

Table 2. Immunogenicity and toxicity of combined DTaP-HepB vaccines with a variety of compositions.

Group	Formulation	DTaP content (μg/ml)	$Al(OH)_3$ (μg Al/ml)	Immunogenicity				BWD toxicity (BWD U/ml)
				D (Unit/ml, Potency)	T (Unit/ml, Potency)	aP (Unit/ml, Potency)	HBsAg (mIU/ml, GM anti-HBsAg IgG titer)	
Alum content	I	236	350	2	4	12.2	200	13.6
	II		500	2	4	13.5	390	21.0
	III		800	2	4	14.0	470	34.2
DTaP content	IV	156	800	2	4	16.4	280	16.6
	V	193		2	4	16.9	355	24.2
	VI	236		2	4	15.1	455	38.7

Table 3. Immunogenicity of combined DTaP-HepB vaccines with different tetanus content according to the storage period of vaccine.

Group	Storage period of vaccine (Weeks)	Immunogenicity			
		D (Unit/ml, potency)	T (Unit/ml, potency)	aP (Unit/ml, potency)	HBsAg (mIU/ml, GM anti-HBsAg IgG titer)
5 Lf/ml of tetanus	0	2	4	12.5	252
	2	2	4	14.7	327
	6	2	4	15.1	321
	11	2	4	13.4	315
	16	2	4	12.7	334
15 Lf/ml of tetanus	0	2	4	14.7	470
	2	2	4	13.5	505
	6	2	4	12.1	247
	11	2	4	14.9	190
	16	2	4	15.5	112

355, and 455 mIU/ml for 156, 193, and 236 μg/ml, respectively (Table 2). This implies that the HBsAg immune response may be related to the DTaP content in the combined DTaP-HepB vaccine, which will be further explained in the below.

Effect of Tetanus Toxoid Content on Stability of Immunogenicity of Combined DTaP-HepB Vaccines

The long-term stability of each component in a combined DTaP-HepB vaccine is very important. Here, the focus was the stability of the immunogenicity of each component in the combined DTaP-HepB vaccine during a storage period at 4°C, together with interrelationship between the immunogenicity and the tetanus content.

Two combined DTaP-HepB vaccines were prepared with different concentrations of the tetanus toxoid, i.e. 5 and 15 Lf/ml of the tetanus toxoid, respectively, in a formulation including 50 Lf/ml of the diphtheria toxoid, 12 μg PN/ml of acellular pertussis, 24 μg/ml of HBsAg, and 800 μg Al/ml of an $Al(OH)_3$ gel. Subsequently, each immunogenicity was chased for 16 weeks (Table 3). Surprisingly, the higher concentration of the tetanus toxoid exhibited an adverse effect on the HBsAg immunogenicity (Figs. 1 and 2), while the potencies of components other than HBsAg remained stable. In the case of the formulation with 15 Lf/ml of the tetanus toxoid, the GM anti-HBsAg IgG titer remained within a range of 470 to 505 mIU/ml for two weeks of storage period, however, the titer dropped sharply to below 250 mIU/ml at week 6 and then gradually decreased to around 100 mIU/ml. On the case of the formulation with 5 Lf/ml of the tetanus toxoid, however, the GM anti-HBsAg IgG titer remained stable within a narrow range of 252 to 334 mIU/ml after 16 weeks of storage.

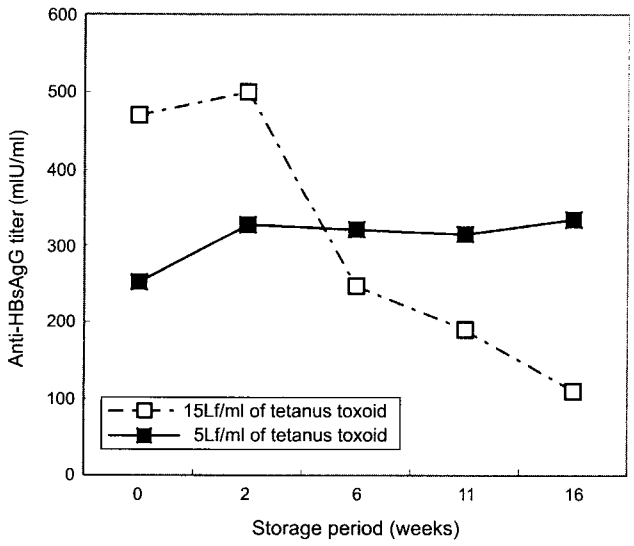

Fig. 1. Effect of the tetanus toxoid content in combined DTaP-HepB vaccines on anti-HBsAg IgG titer.

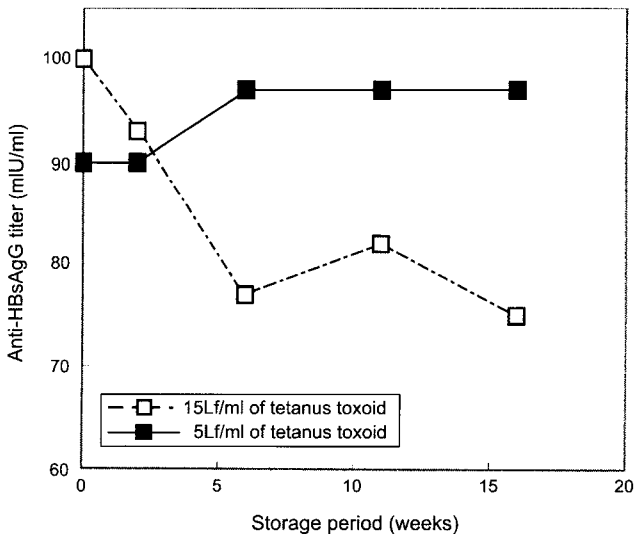

Fig. 2. Effect of the tetanus toxoid content in combined DTaP-HepB vaccines on protective anti-HBsAg antibody rate.

The protective anti-HBsAg antibody rate (percentage of mice above 10 mIU/ml of serum anti-HBsAg IgG as antibody level for HepB virus protection) was also investigated for the same period, and its trend was found to be very similar to that of the GM anti-HBsAg IgG titer (Fig. 2). The protective anti-HBsAg antibody rate for the formulation with 5 Lf/ml of the tetanus toxoid was distributed above 90%. Yet, for the formulation with 15 Lf/ml of the tetanus toxoid, the protective anti-HBsAg antibody rate decreased sharply from an initial 100% to a final 75%. Therefore, these results suggest that, in the combined DTaP-HepB vaccine, the tetanus toxoid or impurities in the tetanus toxoid bulk had a very crucial effect on the stability of the HBsAg immunogenicity in mice

New DTaP-HepB Formulation with Low BWD Toxicity and Stable HBsAg Immunogenicity

Since the results showed that the DTaP and $Al(OH)_3$ gel contents in the DTaP-HepB vaccine were related to the mouse BWD toxicity, and that the tetanus toxoid content was one of determinant factors in stabilizing the HBsAg immunogenicity for a longer storage period, a new formulation was prepared that included 30 Lf/ml of the diphtheria toxoid, 5 Lf/ml of the tetanus toxoid, 10 μg PN/ml of acellular pertussis, 24 μg/ml of HBsAg bulk, and 500 μg Al/ml of an $Al(OH)_3$ gel. This new combined DTaP-HepB vaccine was assayed for its mouse BWD toxicity and long-term HBsAg immunogenicity. The result showed that the BWD toxicity was 8.6, which satisfies the K-FDA criteria for a combined DTaP vaccine (*The minimum requirements for biological products of Korea, K-FDA*), while the anti-HBsAg IgG titer remained between 270 and 310 mIU/ml for 12 weeks (270 mIU/ml at initial time, 325 mIU/ml at week 6, and 310 mIU/ml at week 12). In addition, the potencies of the diphtheria toxoid, tetanus toxoid, and acellular pertussis in this new formulation also met K-FDA criteria of a combined DTaP vaccine.

DISCUSSION

Diphtheria, tetanus, and pertussis are globally distributed diseases, which are still the cause of much suffering for infected people. Consequently, there is an urgent need for the development of a combined DTaP-HepB vaccine that can reduce the cost and enable to vaccinate against all three diseases more conveniently and efficiently.

The major problem in developing a combined DTaP-HepB vaccine is the lower immunogenicity of HBsAg compared to a separately injected case (simultaneous injection of DTaP vaccine and HepB vaccine). However, we found in the present study that the low HBsAg immunogenicity of a combined DTaP-HepB vaccine was apparently due to enzymes or other impurities in the tetanus toxoid bulk. Two formulations with different tetanus toxoid contents were compared by Western blot analysis and the immunogenicity of HBsAg over a 16-week storage period. The analysis of the Western blot pattern did not reveal any HBsAg band alterations, such as appearance of a new band and degradation of bands, regardless of the tetanus toxoid content (figure not shown). Yet, the HBsAg immunogenicity with the higher tetanus toxoid content gradually decreased as the storage period increased, as mentioned in Results. This means that the decreased HBsAg immunogenicity was most likely induced by minute changes, such as damage to the antigenic determinants of HBsAg, or possibly caused by enzymes or other impurities in the tetanus toxoid bulk. Therefore, the current results suggest that highly purified bulks, especially for the tetanus toxoid, should be used to create a combined DTaP-HepB vaccine with stable HBsAg immunogenicity.

It was also very interesting to note that the toxicity in the combined DTaP-HepB vaccine evaluated in the present study was proportionally correlated to the DTaP content. Some researchers have reported on the possibility of incomplete detoxification and partial reversion in the case of detoxifying toxins by formalin alone [11, 16]. This implies that more than one toxoid in a combined DTaP-HepB vaccine may be incompletely inactivated or partially reactivated into a toxin. As such, to prevent the reversion of toxoids, modified methods, such as formalin-lysine detoxification and glutaraldehyde detoxification, have been developed [10, 11, 16, 18]. To eliminate the possibility of toxicity due to incomplete detoxification and toxoid reversion, the use of toxoids produced by modified detoxification methods would seem to be necessary, and this will be further investigated in the future.

In the current study, a higher DTaP content had a positive effect on the HBsAg immunogenicity in the combined DTaP-HepB vaccine (Table 2). Warren and Chedid demonstrated that compounds such as lipopolysaccaride and pertussis toxin present in the pertussis vaccine can play a role as an adjuvant [19]. Therefore, one possible explanation for the stimulation of HBsAg immunogenicity was that the DTaP (more than one component in DTaP) in the combined DTaP-HepB vaccine acted as an adjuvant.

Generally, an alum gel is used to enhance the humoral immune response of component vaccines [7, 19]. This was reconfirmed by our present observation that the combined DTaP-HepB vaccines with a higher alum gel concentration induced stronger HBsAg immune responses (Table 2). Unfortunately, the higher alum gel concentration in the combined DTaP-HepB vaccine also led to the stimulation of mouse BWD toxicity.

In conclusion, to develop a combined DTaP-HepB vaccine with a low toxicity and stable HBsAg immunogenicity, the contents of tetanus toxoid, DTaP, and alum gel concentration are all very important factors to be considered.

REFERENCES

1. Andre, F. E. 1999. Development and clinical application of new polyvalent combined paediatric vaccines. *Vaccine* **26:** 1620–1627.
2. Cooper, P. D. 1994. The selective induction of different immune responses by vaccine adjuvants, pp. 125–158. *In* Ada, G. L. (ed.), *Strategies in Vaccine Design*, Landes Company, Austin, U.S.A.
3. Corbel, M. J., E. Griffiths, and R. Winsnes. 1997. Workshop on Standardisation of Aluminium Adsorbed Vaccines. *Biologicals* **25:** 351–353.
4. Greenberg, D. P., V. K. Wong, S. Partridge, S. J. Chang, J. Jing, B. J. Howe, and J. I. Ward. 2000. Immunogenicity of a *Haemophilus influenzae* type b-tetanus toxoid conjugate vaccine when mixed with a diphtheria-tetanus-acellular pertussis-hepatitis B combination vaccine. *Pediatr. Infect. Dis. J.* **19:** 1135–1140.
5. Gupta, R. K., E. H. Relyveld, E. B. Lindbald, B. Bizzini, S. Benefraim, and C. K. Gupta. 1993. Adjuvants - a balance between toxicity and adjuvanticity. *Vaccine* **11:** 293–306.
6. Gupta, R. K., B. E. Rost, E. Relyveld, and G. R. Siber. 1995. Adjuvant properties of aluminum and calcium compounds, pp. 229–248. *In* Powell, M. F. and Newman, M. J. (eds.). *Vaccine Design: The Subunit and Adjuvant Approach.* Plenum Publishing Corporation, New York, U.S.A.
7. Gupta, R. K. and G. R. Siber. 1995. Adjuvants for human vaccines - current status, problems and future prospects. *Vaccine* **13:** 1263–1276.
8. Iwasa, S. and H. Fujiwara. 1990. Comparison of the detoxification courses of histamine-sensitizing, lymphocytosis-promoting and CHO cell-clustering activities in the process of the production of acellular pertussis vaccine. *Biologicals* **18:** 127–130.
9. Jenson, O. M. and C. Koch. 1988. On the effect of $Al(OH)_3$ as an immunological adjuvant. *Acta Pathol. Microbiol. Immun. Scand.* **96:** 257–264.
10. Kim, S.-Y., H.-S. Kang, Y.-S. Kim. 2000. Phylogenetic analysis of Hepatitis B virus genome isolated from Korean patient serum. *J. Microbiol. Biotechnol.* **10:** 823–828.
11. Linggood, F. V., F. S. Muriel, A. J. Fulthorpe, A. J. Woiwod, and C. G. Pope. 1962. The toxoiding of purified diphtheria toxin. *Brit. J. Exp. Path.* **14:** 177–188.
12. Majgaard, J. O. and C. Koch. 1988. On the effect of $Al(OH)_3$ as an immunological adjuvant. *APMIS* **96:** 257–264.
13. Masood, H., J. L. White, and S. L. Hem. 1994. Relationship between protein adsorptive capacity and the X-ray diffraction pattern of aluminium hydroxide adjuvants. *Vaccine* **12:** 187–189.
14. Park, J.-H., E.-W. Cho, D. G. Lee, J.-M. Park, Y.-J. Lee, E.-A. Choi, and K. L. Kim. 2000. Receptor-mediated endocytosis of Hepatitis B virus preS1 protein in EBV-transformed B-cell line. *J. Microbiol. Biotechnol.* **10:** 844–850.
15. Pines, E., M. Barrand, P. Fabre, H. Salomon, C. Blondeau, S. C. Wood, and A. Hoffenbach. 1999. New acellular pertussis-containing paediatric combined vaccines. *Vaccine* **26:** 1650–1656.
16. Scheibel, I. and C. P. Elo. 1965. Irreversible detoxification of purified diphtheria toxin. *Acta Path. Microbiol. Scand.* **65:** 117–128.
17. Sesardic, D., C. S. Dawes, K. Mclellan, Z. Durrani, S. E. Yost, and M. J. Corbel. 1999. Non-pertussis components of combination vaccines: Problems with potency testing. *Biologicals* **27:** 177–181.
18. Tan, L. U., R. E. Fahim, G. Jackson, K. Phillips, P. Wah, D. Alkema, G. Zobrist, A. Herbert, L. Boux, and P. Chong. 1991. A novel process for preparing an acellulat pertussis vaccine composed of nonpyrogenic toxoids of pertussis toxin and filamentous hemagglutinin. *Mol. Immunol.* **28:** 251–255.
19. Warren, H. S. and L. A. Chedid. 1988. Future prospects for vaccine adjuvants. *CRC Critical Reviews in Immunology* **8:** 83–101.
20. Yoo, Y. C., S. Y. Park, K. B. Lee, and I. Azuma. 2000. MDP-Lys(L18), a synthetic muramyl dipeptide derivative, enhances antitumor activity of an inactivated tumor vaccine. *J. Microbiol. Biotechnol.* **10:** 399–404.